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Recent studies from our laboratory have demonstrated that parathyroid hormone related protein (PTHrP) is necessary for mammary gland development. Our studies have suggested that PTHrP is involved in regulating epithelial-stromal interactions during embryonic mammary development and during adolescent ductal morphogenesis. It is our working hypothesis that PTHrP is an epithelial signal that acts to modulate the ability of mammary stromal cells to support epithelial morphogenesis. In the second year of this project, we have gathered additional evidence that PTHrP plays a critical role in the control of mammary stromal cell function. We have shown that PTHrP is the signal from the mammary epithelium that necessary for the proper differentiation of the mammary mesenchyme. In addition, we have also shown that mammary stromal cells must express the PPR1 in order to support mammary epithelial morphogenesis, thereby conclusively demonstrating that stromal cells are the critical target for PTHrP's actions in the mammary gland. Finally, we have begun to identify potential downstream signaling partners to PTHrP in mammary stromal cells in order to elucidate the mechanisms by which PTHrP modulates stromal cell function.

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Maureen E. Dunbar 7/29/99

PI - Signature Date

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Introduction

Recent studies from our laboratory have demonstrated that parathyroid hormone related protein (PTHrP) is necessary for mammary gland development (1,2). Our studies have suggested that PTHrP participates in the regulation of epithelial-mesenchymal interactions during embryonic mammary development and also during adolescent ductal morphogenesis. The purpose of this project is to elucidate the mechanisms by which PTHrP regulates epithelial-mesenchymal interactions in the developing mammary gland. Specifically, it is our hypothesis that PTHrP is an epithelial signal that acts on mammary stromal cells and modulates their ability to support epithelial morphogenesis. In the first year of this proposal we demonstrated that PTHrP is expressed in epithelial cells, and the PTH/PTHrP receptor (now referred to as PPR1) is expressed in the surrounding stroma throughout development (3). In addition we demonstrated that the expression of PTHrP and PPR1 is the most prominent in the embryonic mammary bud and in the terminal end bud during adolescence. These data are interesting and suggest that PTHrP and PPR1 are expressed in regions of the mammary gland that are actively proliferating and undergoing ductal morphogenesis. In the second year of this project, we have gathered further evidence to support our hypothesis that PTHrP regulates stromal cell function during mammary development. In addition, we have begun to elucidate some of the potential downstream signaling partners to PTHrP in the mammary gland. The following sections report our progress over the second year of this project.

Body

Objective 1: Temporal-spatial expression of PTHrP and PPR1 during mammary gland development

The goal of technical objective 1 was to define the pattern of PTHrP and the PTH/PTHrP receptor (now referred to as PPR1) during mammary gland development. The experiments proposed under objective 1 were completed in year one of this project and the specifics of these experiments were discussed in last years annual report. Summarizing this data, we have found that PTHrP is expressed in mammary epithelial cells while PPR1 is expressed in the surrounding stromal cells throughout mammary gland development. The most intense expression of PTHrP and PPR1 are in the embryonic mammary bud and in the terminal end buds during puberty. This pattern of expression is interesting because it suggests that PTHrP and PPR1 expression are the most intense in regions of the mammary gland that are actively proliferating and undergoing ductal morphogenesis. The results from these experiments have been published *in Developmental Biology* and a re-print of this article has been included in the appendices of this report (3).

Technical Objective 2: Effects of PTHrP on growth factor production by mammary stromal cells

The central hypothesis underlying this proposal is that PTHrP affects epithelial ductal branching morphogenesis by regulating mammary stromal cell function. In the past year we have gathered more evidence to support a critical role for PTHrP in the control of mammary stromal cell function during embryonic development. Before discussing this new data, it will be useful to review the embryonic development of the mammary gland (4). A schematic diagram of this process is shown in Fig. 1. In mice, mammary development begin on E10 with the formation of five pairs of mammary buds which are thickenings of the epidermis along an imaginary line, known as the milk line, that extends from the anterior to the posterior limb buds. The mammary buds are fully formed by E12 and are composed of a bell shaped mammary epithelial bud surrounded by a dense mammary mesenchyme.

An important aspect of embryonic mammary development is the sexual dimorphism that occurs after the formation of the mammary bud (4) (see Fig. 1). In female mice, the mammary buds remains quiescent until E16 when they undergo a transition into the second step of embryonic development, the formation of the rudimentary ductal tree. This process involves the elongation of the mammary bud, its penetration into the mammary fat pad precursor and the initiation of branching morphogenesis. By birth, the mammary gland consists of 15-20 branching epithelial ducts contained within a mammary fat pad. This initial pattern persists until puberty, at which time the mature virgin gland is formed through a second round of branching morphogenesis, regulated by circulating hormones.

In male mice, on E13 the fetal testes begin to produce androgen which leads to the destruction of the mammary buds. In response to androgens, the mammary mesenchyme condenses around the neck of the mammary bud, and by E14, severs its connection to the epidermis. The remaining epithelial cells subsequently degenerate by birth. This process has been studied in some detail and it is known to rely on a series of epithelial-mesenchymal interactions (4). First, androgen receptor expression is induced in the mammary mesenchyme between E12 and E14 under the direction of mammary epithelial cells (5). The mesenchymal cells are the cells that subsequently respond to fetal androgens, and they in turn sever the epithelial stalk and destroy the epithelial cells (4,6,7). Although the molecular details of this process have not been elucidated, it is clear from the existing literature that epithelial cells and mesenchymal cells cooperate with one another in the destruction of the mammary bud in response to androgens (4-7).

In the absence of PTHrP there are two major defects in embryonic mammary development. As summarized in the original proposal, we found that ablation of the PTHrP gene leads to a failure of mammary development at the transition of the mammary bud into the initial phase of branching morphogenesis. These data have been published in *Development* and a reprint of this manuscript is included in the appendices (1). In addition to the

compete failure of mammary epithelial development in female PTHrP-knockouts, there is also a loss of the normal sexual dimporhism between male and female mice. In examining the mammary buds in PTHrP and PPR1-knockout male embryos at E15, we were suprised to notice that the mammary buds do not degenerate on E14-E15 as in the wild type littermates. The histological findings in these embryos are shown in Fig. 2. Fig 2A shows the typical appearance of a wild type female mammary bud at E15. One can see the nice, bell shaped mammary bud surrounded by the dense mammary mesenchyme. In contrast, at E15, the wild type male bud is actively being destroyed (Fig. 2B). One can see that the mesenchyme had condensed around the neck of the bud and the mammary epithelial cells in this region have degenerated. However, in PTHrP and PPR1 knockout embryos (Fig. 2C,D) at E15, the mammary buds resemble those in wild type females. There is a complete lack of the androgen mediated mesenchymal condensation and the neck of the mammary bud is well preserved. As a result, the mammary buds in male and female PTHrP-knockout embryos are indistinguishable at E15. The mutant buds persist until E16-17, at which point they fail to undergo the initial round of branching morphogenesis and instead degenerate, findings identical to that previously reported for female PTHrP- and PPR1-knockout mammary rudiments (1).

The destruction of the mammary bud in male embryos is an example of programmed cell death (Fig.3). In the wild type male bud at E15 (Fig. 3A) there is widespread TUNEL staining in the region of the degenerating epithelial stalk. This appears to involve both epithelial and mesenchymal cells. In contrast, in PTHrP-knockout male embryos (Fig. 3B) there is no apoptosis. Similar results were obtained in PPR1-knockout embryos and, in both strains of knockout mice, the lack of TUNEL staining was identical to the results obtained with wild type-female embryos (8). Therefore, in the absence of PTHrP or PPR1, the pattern of sexual dimporhism normally observed during early mammary development is abolished.

The androgen mediated destruction of the mammary bud is dependent on the presence of functional androgen receptors in the dense mammary mesenchyme, and the expression of these signals is induced by the mammary epithelium. The absence of the androgen response in the PTHrP- and PPR1-knockout buds combined with the epithelial expression of PTHrP and the mesenchymal expression of PPR1, led us to hypothesize that PTHrP might be the epithelial signal responsible for the induction of androgen receptor expression in the mesenchyme. To investigate this possibility, we examined androgen receptor expression in the mammary buds of wild type female and male and female PTHrP- and PPR1-knockout embryos at E15 by immunohistochemistry. As seen in Fig. 4B , in wild type bud there is strong staining for androgen receptor in the nucleus of the cells comprising the dense mammary mesenchyme. In contrast, this staining is absent in the PTHrP- (Fig. 4A) and PPR1-knockout (Fig. 4C) buds. In these glands there are only occasional nuclei that stain weakly for androgen receptor within the mesenchymal cells. The absence if androgen receptor staining appears to be

specific for the mammary mesenchyme for androgen receptor staining is normal within the testes of knockout mice (9).

Androgen receptor expression is one of the characteristics of the mammary mesenchyme that sets it apart from the dermal mesenchyme (4). Therefore the absence of androgen receptor expression in the PTHrP- and PPR1-knockouts suggested that there might be more fundamental defects in the differentiation of these cells. The other classic marker of the specialized mammary mesenchyme is tenascin C (4). Tenascin C is a heparin sulfate proteoglycan that is present within the extracellular matrix of the condensing mesenchyme associated with several developing organs. We next examined PTHrP- and PPR1-knockout mammary buds for the expression of tenascin C by immunohistochemistry. As shown in Fig. 4, the results from these experiments were similar to those with androgen receptor expression. The wild type epithelial bud (Fig. 4E) was surrounded with a halo of tenascin C within the extracellular matrix of the dense mammary mesenchyme, but not within the general dermal mesenchyme. In contrast, there was no tenascin C staining surrounding the PTHrP- (Fig. 4D) and PPR1-knockouts (Fig. 4F), suggesting that, in the absence of PTHrP or PPR1, the dense mammary mesenchyme does not differentiate properly. As with androgen receptor, there did not appear to be a generalized defect in tenascin C expression, as there was ample tenascin C staining in the developing bones of knockout embryos (10).

These results are interesting on two levels. First, they lend support to our hypothesis that PTHrP contributes to the differentiation of the dense mammary mesenchyme, as both classical markers of this phenotype are absent in these cells in the absence of PTHrP. Second, these results provide a mechanistic explanation for the failure of the androgen-mediated destruction of the knockout mammary buds. That is, PTHrP appears to be the epithelial signal that induces androgen receptor expression within the mesenchymal cells, and without PTHrP the mesenchymal cells fail to express this receptor and become deaf to the androgen signal.

These experiments were not part of the original proposal as they followed from data that was unknown at the time of submission. As a result they are not part of the original statement of work. However, we feel that these experiments are related to the overall goal of this project and that the results are important in understanding completely the role of PTHrP in mammary development. These results have recently been published in *Development* and a copy of this article has been included in the appendices of this report (8).

Another series of experiments we performed to test our hypothesis that the mammary stroma is the critical target for PTHrP's actions in the mammary gland were tissue recombination and transplantation experiments using mammary epithelial buds and mammary mesenchyme from wild type and PPR1 knockout embryos. In these experiments, knockout and wild type mammary epithelial buds and mammary mesenchyme were recombined in all possible recombinations (see Table 1) and grown under the kidney capsule of recipient females. We reasoned that if our hypothesis was valid, and PTHrP and PPR1 do represent an epithelial-mesenchymal signaling circuit, the PPR1-null

phenotype would be expected to segregate with mesenchymal tissue. This is, receptor knockout mesenchyme should not be able to support the outgrowth of either receptor knockout or normal epithelial buds, but receptor knockout epithelium should be able to form ducts when combined with normal mesenchyme.

Table 1 and Fig 5. summarize the results of these experiments. As expected, wild type epithelial buds paired with wild type mammary mesenchyme (wt-MGE + wt-MGM) consistently gave rise to a series of branched epithelial ducts contained within a fatty stroma (Fig. 5A). In contrast, ductal outgrowth was never detected when PPR1-knockout epithelial buds were paired with PPR1-knockout mesenchyme (KO-MGE + KO-MGM) (Fig.5B). These transplants gave rise to a fatty stroma that was devoid of mammary epithelial cells, reproducing the phenotype of the PPR1-knockout embryos. Recombinations consisting of PPR1-knockout epithelium paired with wild type mesenchyme (KO-MGE + wt-MGM) uniformly gave rise to branched epithelial ducts within a fatty stroma (Fig.5C). However, although the receptor knockout epithelial buds consistently grew out and formed a rudimentary branching ductal structure, the growth of the resulting ducts appeared stunted as compared to ducts from wild type epithelium paired with wild type mesenchyme. Nonetheless, the PPR1 knockout epithelial cells survived and had the capacity to initiate branching morphogenesis when paired with normal mesenchyme. All recombinants composed of wild type epithelium paired with PPR1 knockout mesenchyme (wt-MGE + KO-MGM) lacked any evidence of epithelial ductal outgrowth and consisted of fatty stroma alone (Fig.5D). These results suggest that PPR1 knockout mesenchyme was unable to support the survival or morphogenesis of normal epithelial cells in this transplant system. In addition these results demonstrate that the defects in mammary epithelial cell morphogenesis and survival seen in the PPR1 knockout mice segregate with mesenchymal tissue and demonstrate conclusively that the mesenchyme is the critical target for the actions of PTHrP during ductal mammary morphogenesis.

Although these experiments were also not part of the original statement of work, these experiments are related to the overall goal of this project and we feel that the results from these experiments help to strengthen our hypothesis that PTHrP modulates stromal cell function during mammary development. These results have been published in *Developmental Biology* (3, see attached re-print).

Now that we have conclusive evidence that the mesenchyme or stroma is the target for PTHrP's actions in the mammary gland, we next wanted to test our hypothesis that PTHrP regulates ductal morphogenesis by modulating stromal cell function. To investigate this possibility we proposed two technical objectives that were designed to identify potential downstream signaling partners to PTHrP in mammary stromal cells. Under technical objective 2, we designed experiments to examine the affects of PTHrP on three candidate factors that we felt were potential downstream agents of PTHrP's actions. These three growth factors hepatocyte growth factor (HGF/SF), insulin-like growth factor (IGF-1), and keratinocyte growth factor (KGF). Under technical

objective 3, we proposed to perform subtractive hybridization on primary cultures of mammary stromal cells to define more comprehensively the changes in stromal cell gene expression following PTHrP signaling.

We first chose to examine the effects of PTHrP on the expression of HGF/SF in our mammary stromal cell cultures. As shown in our annual report for year one of this project, we have not found any evidence that PTHrP altered HGF/SF mRNA levels in our mammary stromal cell cultures. It has recently been reported that HGF/SF-knockout mice do not have defects in embryonic mammary development, and therefore do not phenocopy our PTHrP-knockout mice (11). These results suggest that HGF/SF does not act downstream of PTHrP during embryonic mammary development, and therefore support to our findings that PTHrP does not regulate HGF/SF expression in mammary stromal cells. We have also proposed to examine the expression of IGF-1 and KGF in mammary stromal cells treated with PTHrP. However, it has recently been reported that KGF-knockout mice (12) and IGF-1-knockout mice (13) also do not have a mammary phenotype and once again do not phenocopy the PTHrP-knockout. Based on these findings we feel that it is highly unlikely that these growth factors act downstream of PTHrP during embryonic mammary development, and have decided not to pursue examining the effects of PTHrP on KGF and IGF-1 levels in our mammary stromal cell cultures. Given the multitude of possible molecules that might act downstream of PTHrP, we have decided against trying to compile a list of other potential candidates that might act downstream of PTHrP in mammary stromal cells. Instead chosen to focus our attention on trying to define comprehensively the changes in mesenchymal gene expression elicited by PTHrP.We have These experiments are discussed in detail under technical objective 3.

As mentioned earlier, we have found that the expression of PTHrP and PPR1 is very intense in the terminal end buds, which are the sites of active cellular proliferation and differentiation of the mammary ducts during adolescence. This finding is interesting because it suggests that PTHrP and PPR1 might be involved in the regulation of ductal morphogenesis during adolescent growth of the mammary gland. Consistent with this hypothesis, PTHrP-overexpressing mice have severe defects in ductal morphogenesis during puberty (2). Therefore, it is possible that, in addition to playing a role in embryonic mammary development, PTHrP also plays a role in the hormonally induced ductal morphogenesis that occurs during puberty. In the second part of technical objective 2 we proposed to examine the expression of growth factors in our stromal cell cultures in response to PTHrP in the presence of exogenously added estrogen and progesterone. We have not yet started these experiments and are thus slightly behind schedule with repect to our statement of work. We plan to initiate these experiments in the next month. In addition we also proposed to determine the effects of PTHrP on the estrogen and progesterone stimulated growth of mammary epithelial cells. As outlined in the original statement of work, we are scheduled to begin these experiments in the next few months.

Objective 3: Characterization of differentially expressed genes in embryonic mammary buds from normal and PTHrP null mice

The main goal of technical objective 3 is to define comprehensively the changes in gene expression in mammary stromal cells following PTHrP signaling. We originally proposed to perform mRNA differential display on RNA isolated from embryonic mammary buds from normal and PTHrP-null mice in order to identify genes that are differentially expressed in these mice. As mentioned in last years report, due to several technical difficulties we were having with the differential display on the microdissected mammary buds, we have decided instead to perform subtractive hybridization on cultured mammary stromal cells treated with and without PTHrP to define more comprehensively the changes in stromal cell gene expression elicited by PTHrP. We will then use the knockout embryos as a screen to identify those changes that are functionally important.

We have performed subtractive hybridization on PTHrP-treated and untreated primary cultures of mouse mammary stromal cells. The basic method behind cDNA subtraction was as follows: Mammary stromal cells were treated with 10^{-7} M PTHrP for 6 and 24 hours. The time points 6 and 24 hours were chosen to detect genes that might be directly upregulated by PTHrP signaling (6 hours) and those whose upregulation may be secondary to PTHrP signaling (24 hours). The cells were then harvested and poly A+ mRNA was isolated using the Poly A+ Pure Kit from Ambion. cDNA subtraction was performed using the PCR-Select cDNA Subtraction Kit from Clontech. Briefly, the Poly A+ mRNAs from PTHrP-treated and PTHrP-untreated cells were converted into cDNA populations referred to as tester and driver, respectively. The two cDNA populations were then hybridized, and the hybridized sequences removed thereby eliminating cDNAs that are common to both populations of cells. The unhybridized cDNAs were then amplified by PCR and then blunt end cloned into a PCR-cloning vector and screened by dot blot analysis. For dot blot analysis inserts from the individual clones were amplified by PCR and blotted in groups of 96 onto nylon filters and hybridized to forward- and reverse-subtracted clones. The forward -subtracted probe was made from the same subtracted cDNA library used to construct the library. To make the reverse subtracted probe, subtractive hybridization is performed with the original tester cDNA as driver and the driver cDNA as tester. Clones representing mRNAs that are truly differentially expressed will hybridize only with the forward-subtracted probe; clones that hybridize with the reverse-subtracted probe may be considered background.

We have recently completed the initial subtraction protocol and the initial screening procedure described above and have isolated 26 individual clones that represent genes that may be regulated by PTHrP. These clones were sequenced and the sequences compared against the GenBank database to identify known genes. A complete list of these clones can be found in Table 2. Fourteen of these clones represented previously identified genes that could be assigned to one of five categories: 1) extracellular matrix and associated

molecules 2) transcription/differentiation factors; 3) growth factors; 4) signal transduction proteins; and 5) proteins that are thought to have primarily intracellular functions. The remaining twelve clones are unknowns.

In order to confirm that these clones represent truly differentially expressed genes, we are in the process of performing RNase protection analysis on RNA from PTHrP-treated mammary stromal cells using the subtracted clones as probes. As shown in Fig 6., we have currently assayed 6 of these clones by RNAse protection analysis and have identified one clone that appears to be differentially expressed by PTHrP in mammary stromal cells. This clone represents the gene for colony stimulating factor -1 (csf-1), which is a homodimeric serum growth factor that is involved in regulating mononuclear phagocytes. We find this result interesting for a number of reasons. First, mutant mice that contain a single mutation which inactivates the csf-1 gene have defects in mammary development during pregnancy/lactation similar to the defects seen in our PTHrP-overexpressing mice (2, 14). Second, PTHrP has been shown to regulate csf-1 expression in other systems (15). Finally, csf-1 has been implicated in the development of breast cancer where it appears to regulate tumor cell invasion (16). We are currently in the process of examining csf-1 mRNA levels in our PTHrP-overexpressing mice to determine if it acts downstream of PTHrP during in mammary development during pregnancy/lactation.

We are generally on time with respect to our original statement of work as far as this technical objective is concerned. By the end of the second year we had planned to confirm the differential expression of subtracted clones, which we have successfully done. We had initially planned to use *in situ* hybridization on our knockout embryos as a way to screen genes identified by subtraction. However, because csf-1 is not expressed in the embryonic mammary gland, instead of performing *in situ* hybridization on embryonic mammary buds, we will confirm the differential expression of csf-1 in our PTHrP-overexpressing mice. Also, in our statement of work we had planned to begin sequencing the subtracted clones and searching the Genbank databse in the last year of this project. We have already sequenced and have determined the identity of these clones by searching Genbank and are therefore slightly ahead of schedule with respect to this aspect of the project.

Conclusion

During the second year of this project we have focused our attention on gathering additional evidence that PTHrP plays a critical in the control of mammary stromal cell function during embryonic development. Our most exciting finding during this past year is that it appears that PTHrP is the epithelial signal that is responsible for inducing androgen receptor and tenascin C expression within the dense mammary mesenchyme (8). More significantly, these data suggest that PTHrP is an epithelial signal from the mammary bud that is involved in the cell fate decisions that lead the ventral mesenchyme cells

to become differentiated dense mammary mesenchyme cells, with the ability to support proper epithelial morphogenesis.

We have also provided evidence that PTHrP affects stromal cells function in a way that affects epithelial branching morphogenesis during embryonic mammary development. Through a series of tissue recombination experiments, we have demonstrated that mammary stromal cells must express PPR1 in order to support the outgrowth and morphogenesis of the mammary epithelium (3). These observations prove that PTHrP acts to modulate stromal cells function in a way that is necessary for these cells to support branching morphogenesis.

Finally, we have begun to examine the potential downstream effectors of PTHrP's actions in the mammary gland using the technique of subtractive hybridization on PTHrP-treated and untreated mammary stromal cells. To date, we have identified colony stimulating factor-1 (csf-1) as a potential downstream signaling partner to PTHrP during mammary gland development. Over the next year we will be concentrating our efforts on identifying the molecules that act downstream of PTHrP in stromal cells so that we can define the mechanisms by which PTHrP enables the stroma to support epithelial morphogenesis.

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Table 1- Summary of PTH/PTHrP Receptor-Knockout and Wild-type Recombination Experiments

Mesenchyme	Epithelium	n	Fat only	Ducts
WT	WT	4	0	4
KO	KO	4	4	0
WT	KO	2	0	2
KO	WT	3	3	0

Table 2- List of Clones Identified by Subtractive Hybridization of PTHrP-treated and untreated mamamry stromal cells

Cell Adhesion/Extracellular Matrix
stromelysin 1
PG-M Core Protein
ryudocan (syndecan 4)
type III collagen
fibrillin

Signal Transduction
14-3-3 zeta
ARF-related protein

Transcription factors
LZIP
msx-1 homologue

Growth Factors
colony stimulating factor-1
T1 protein (IL-6 receptor homologue)

Intracellular Calcium Associated Proteins
calpactin
calreticulin
ribophorin

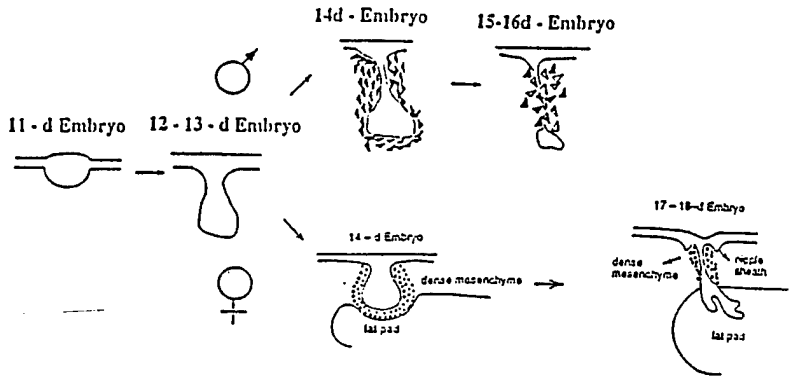

Figure 1. Outline of mammary development. See text for details. Triangles represent mesenchymal cells responding to androgens and males; circles represent mesenchymal cells in females.

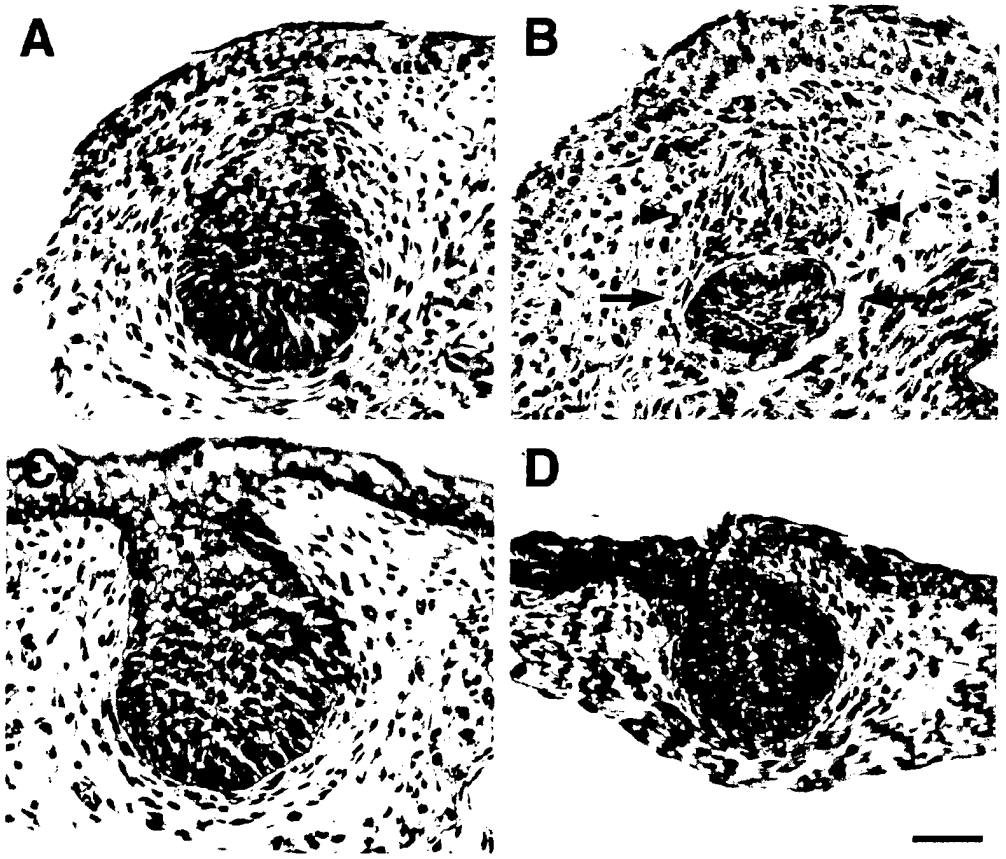

Figure 2. Failure of the androgen-mediated destruction of the mammary buds in PTHrP- and PPR1-knockout embryos. Hematoxylin and eosin-stained sections through E15 mammary buds taken from a wild-type female embryo (A), a wild-type male embryo (B), a PTHrP-knockout male embryo (C), and a PPR1-knockout male embryo (D). In the normal male embryo (B), the mesenchymal cells have condensed around the degenerating epithelial stalk (between arrowheads) and the epithelial remnant is misshapen and disconnected from the epidermis (arrows). In contrast, note the lack of mesenchymal condensation and the well preserved epithelial stalks in C and D. Scale bar represents 11 microns.

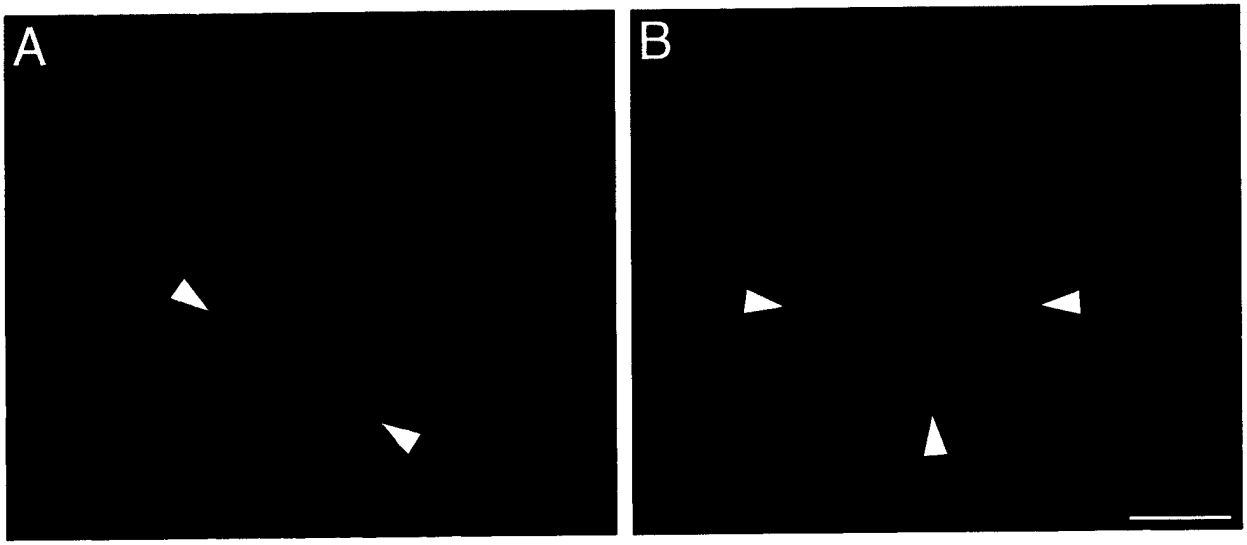

Figure 3. Programmed cell death in male mammary buds at E15. TUNEL assays were performed on sections through mammary buds from (A) a wild-type male embryo (B) and a PTHrP-knockout male embryo. Note the multitude of apoptotic nuclei in the region of the stalk and mesenchymal condensation and in the epithelial remnant (between arrowheads) in the wild-type embryo. In contrast, the mammary bud in the PTHrP-knockout male embryo (outlined by arrowheads) is well preserved and that there is no apoptosis in either the epithelial bud or in the mammary mesenchyme. Scale bar represents 8 microns.

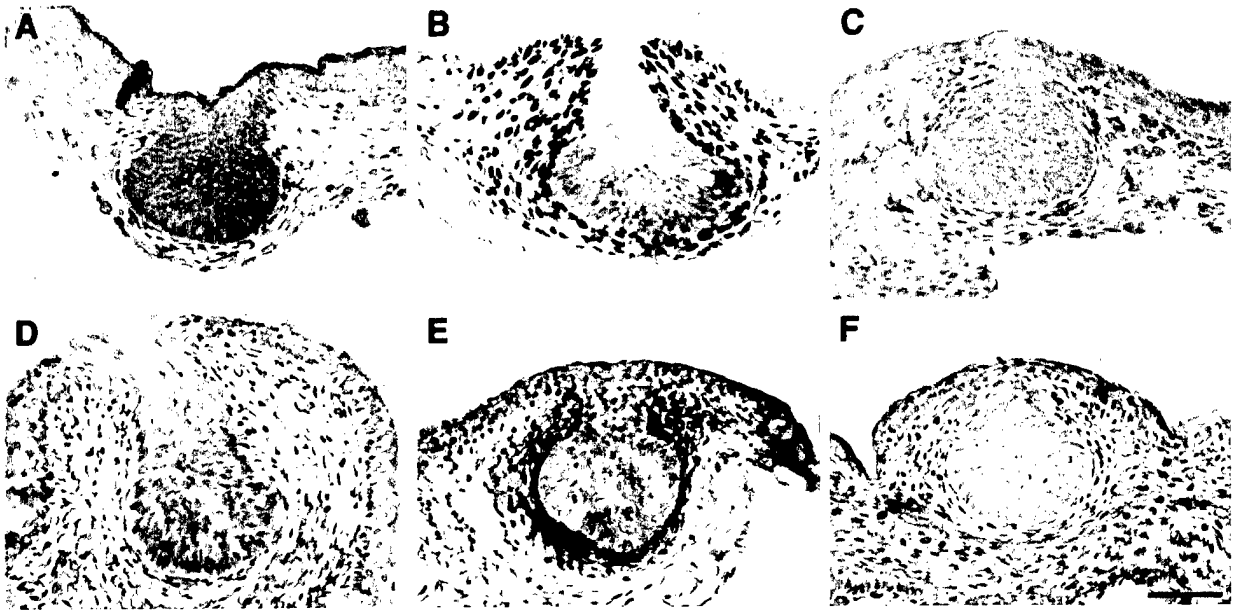

Figure 4. Androgen receptor and tenascin C staining of E15 mammary buds. (A-C) represent sections stained for androgen receptor and (D-F) represent sections stained for tenascin C. (A and D) are sections through male PTHrP-knockout mammary buds. (B and E) are sections through female wild-type buds. (C and F) are sections through male PPR1-knockout mammary buds. Again, note the well preserved mammary buds in the male knockouts. There is prominent nuclear staining for androgen receptor in the dense mammary mesenchyme of the wild-type bud in (B), but little or no androgen receptor staining in the PTHrP-knockout bud in (A) and the PPR-1-knockout bud in (C). Likewise, there is prominent staining for tenascin C in the extracellular matrix surrounding the wild-type bud (E), but an absence of similar staining in the knockouts (D and F).
Scale bar represents 12 microns.

Figure 5. Mesenchymal PPR1 is necessary for the initiation of epithelial outgrowth

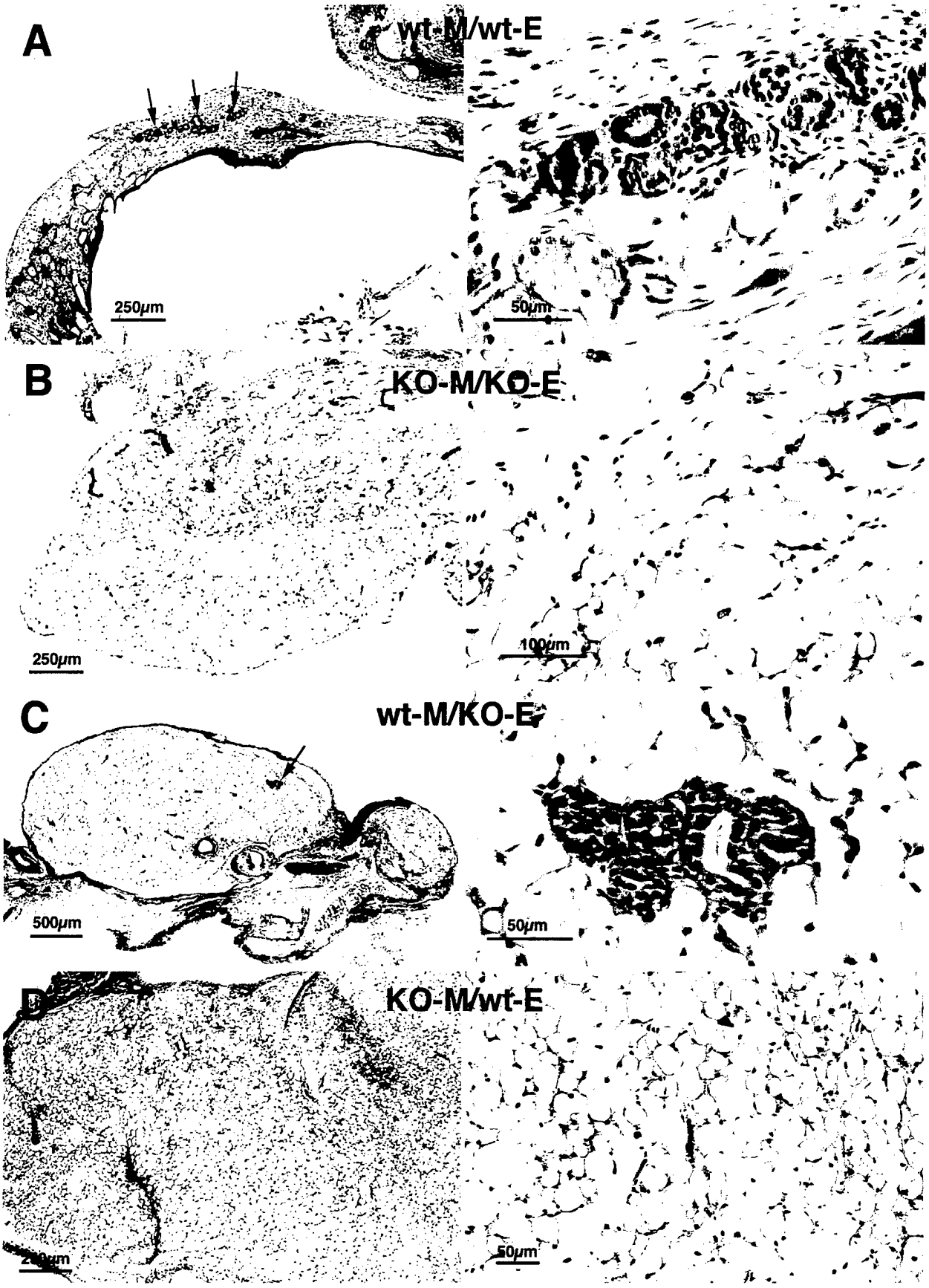

Figure 5. Tissue recombination and transplantation experiments using mammary epithelium and mesenchyme from PTH/PTHrP receptor-knockout embryos. PTH/PTHrP receptor-knockout and wild-type epithelium and mesenchyme were dissected from 13-day-old embryos and recombined in four possible combinations and grown under the kidney capsule of recipient female mice. A. Wild-type epithelium recombined with wild-type mesenchyme (wt-MGE + wt-MGE). B. PTH/PTHrP receptor-knockout epithelium (KO-MGE) paired with PTH/PTHrP receptor-knockout mesenchyme (KO-MGM). C. Wild-type mesenchyme (wt-MGM) paired with PTH/PTHrP receptor-knockout epithelium (KO-MGE). D. Knockout mesenchyme (KO-MGM) paired with wild-type epithelium (wt-MGE). Arrows indicate mammary epithelial ducts. Note that tissue recombinants containing knockout mesenchyme (KO-MGM) failed to form mammary epithelial ducts when grown under the kidney capsule of recipient females.

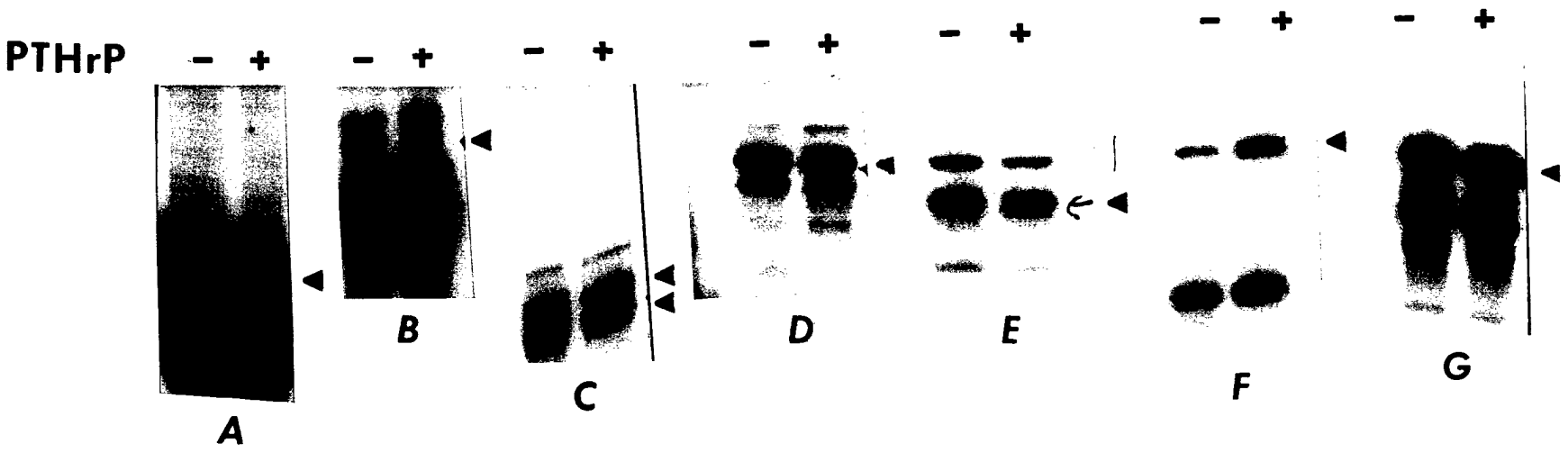

Figure 6. Confirmation of putative differentially expressed cDNAs by RNase protection analysis. RNase protection analysis of 50 ug of total RNA from PTHrP-treated and PTHrP-untreated mammary stromal cells was performed using probes for (A) stromelysin, (B) ryudocan, (C) calreticulin, (D) calpactin, (E) 14-3-3 zeta, (F) csf-1, and (G) GAPDH (which serves as a loading control). Note that all of these clones are expressed in mammary stromal cells, and that csf-1 is upregulated by PTHrP in these cells.

Appendix 1 - List of Key Research Accomplishments

Technical Objective 1 - Temporal and spatial expression of PTHrP and the PTH/PTHrP receptor (PPR1) during mammary gland development

- We have shown by RNase protection analysis and in situ hybridization that PTHrP is expressed in mammary epithelial cells, while PPR1 is expressed in the surrounding stromal cells throughout mammary development.

- The most intense expression of PTHrP and PPR1 is in the embryonic mammary bud and in the terminal end bud during puberty, suggesting that they are involved in active ductal morphogenesis during mammary development.

Technical Objective 2 - Effects of PTHrP on growth factor production by mammary stromal cells

- We have generated primary cultures of mouse mammary stromal cells that express PPR1 and respond to PTHrP with an increase in intracellular cAMP.

- By RNase protection analysis we have demonstrated that PTHrP does not alter HGF/SF mRNA levels in mammary stromal cells.

- We have demonstrated that PTHrP signaling is necessary for sexual dimporhism during embryonic mammary development.

- We have shown that PTHrP induces androgen receptor and tenascin C expression in the dense mammary mesenchyme during embryogenesis, suggesting that PTHrP is involved in the differentiation of the dense mammary mesenchyme.

- Through tissue recombination and transplantation experiments we have shown that mammary stromal cells must express PPR1 in order to support proper mammary ductal morphogenesis.

Technical Objective 3 - Characterization of differentially expressed genes in embryonic mammary buds

- Using subtractive hybridization, we have identified 26 known and 12 unknown genes that may be differentially expressed by PTHrP in mammary stromal cells.

- We have shown by RNase protection analysis that colony stimulating factor-1 (csf-1) expression is upregulated in mammary stromal cells following PTHrP treatment.

Appendix 2 - List of Publications and Abstracts

Invited review articles

1. Dunbar ME and Wysolmerski JJ. (1999) Parathyroid hormone related protein: A developmental regulatory molecule necessary for mammary gland development. *Journal of Mammary Gland Biology and Neoplasia.* 4:21-34

2. Dunbar ME, Wysolmerski JJ, and Broadus AE. (1996). Parathyroid hormone related protein: from hypercalcemia of malignancy to developmental regulatory molecule. *American Journal of the Medical Sciences.* 312:287-294.

Peer-reviewed publications

1. Dunbar ME, Dann PR, Robinson GW, Hennighasuen L, Zhang J-P, and Wysolmerski JJ. (1999) Parathyroid hormone related protein signaling is necessary for sexual dimporhism during embryonic mammary development. *Development*, in press. *

*Please note that this article is currently in press so we haven't yet recieved any re-prints. A copy of the page proofs ahve been included instead.

2. Dunbar ME, Young P, Zhang J-P, McCaughern-Carucci JF, Lanske B, Orloff J, Karaplis A, Cunha G, and Wysolmerski JJ. (1998) Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone related protein. *Developmental Biology* **203:**75-89.

3. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Karaplis A, and Broadus AE. (1998). Rescue of parathyroid hormone related protein knockout mouse demonstrates that parathyroid hormone related protein is essential for mammary gland development. *Development* **125:**1285-1294.

Abstracts

1. Dunbar ME, Dann PR, Dreyer BE, Broadus AE, Philbrick WM and Wysolmerski JJ. Transient early overexpression of PTHrP leads to subsequent defects in mammary development. Oral presentation: The Endocrine Society Annual Meeting, June 12-15. San Diego, California.

Abstracts continued

2. Dunbar ME, Dann PR, Robinson G, and Wysolmerski JJ. PTHrP Signaling is necessary for sexual dimporhism during fetal mammary development. Oral presentation: Second Joint Meeting of the American Society for Bone and Mineral Research and International Bone and Mineral Society, December 1-6, 1998. San Francisco, California.

3. Dunbas ME, Young P, Zhang J-P, Orloff JJ, Karaplis A, Cunha G, anf Wysolmerski JJ. Stromal cells are critical targets in the regualtion of mammary ductal morphogenesis by PTHrP. Poster presentation: Second Joint Meeting of the American Society for Bone and Mineral Research and International Bone and Mineral Society, December 1-6, 1998. San Francisco, California.

4. Dunbar ME, McCaughern-Carucci JF, Zhang J-P, Orloff JJ, and Wysolmerski JJ. The PTH/PTHrP receptor is expressed in mammary stromal cells throughout development. Poster presentation. The American Society for Cell Biology Annual Meeting, December 12-15, 1997. Washington DC.

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TRANSIENT EARLY OVEREXPRESSION OF PTHrP LEADS TO SUBSEQUENT DEFECTS IN MAMMARY DEVELOPMENT.

M Dunbar*, P Dann, B Dreyer, A E Broadus, W M Philbrick, J J Wysolmerski. Internal Medicine, Yale University School of Medicine, New Haven, CT.

PTHrP participates in epithelial-mesenchymal interactions during the development of several organs, including the mammary gland. Overexpression of PTHrP directed by a keratin-14 (K14) promoter results in a severe inhibition of ductular proliferation and branching morphogenesis during puberty and the lack of PTHrP or the PTH/PTHrP receptor leads to a complete failure of the initiation of ductular branching morphogenesis during embryonic development. We have shown that the knockout phenotypes are the result of a failure to condition the mammary mesenchyme properly. Therefore, we asked if the changes resulting from PTHrP overexpression might also be a consequence of early changes during prepubertal development, or if they were dependent on the continued overexpression of PTHrP during puberty. To approach this question, we designed a tetracycline-regulated ("tet-off") K14-PTHrP transgenic mouse. We generated two strains of transgenic mice, one in which tet transactivator expression was driven by the K14 promoter (K14-tTA) and one in which PTHrP (1-141) expression was driven by a tet operon-regulated minimal promoter (pTet-PTHrP). The binary system effectively targeted transgene expression to mammary epithelial cells and was fully repressed by tetracycline. We examined the mammary gland morphology of four groups of double transgenics: mice always overexpressing PTHrP, mice overexpressing PTHrP only before puberty (up to 4 weeks of age), mice overexpressing PTHrP only during puberty (after 4 weeks of age) and mice never overexpressing PTHrP. Mice were sacrificed at 8 weeks of age. Those never seeing overexpression were identical to wild-type controls, and those always overexpressing PTHrP had severe defects in ductular proliferation and branching identical to the original K14-PTHrP mice. Mice overexpressing PTHrP before puberty but not during puberty had the same severe defects in both ductal proliferation and branching seen in the original K14-PTHrP mice. In contrast, mice overexpressing PTHrP during puberty, but not before, had no defects in ductual branching and had only a mild impairment of ductular proliferation. Therefore, pre-pubertal, but not concurrent overexpression of PTHrP is necessary and sufficient to cause the severe pubertal mammary defects seen in K14-PTHrP transgenic mice. Based on these observations, we propose that early overexpression of PTHrP reprograms the mammary stroma, impairing its ability to direct subsequent epithelial morphogenesis. The ability of PTHrP to affect mesenchymal cell differentiation is likely central to its ability to direct morphogenesis in the mammary gland and at other sites of epithelial-mesenchymal interaction.

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PTHrP Signaling is Necessary for Sexual Dimorphism During Fetal Mammary Development. M. Dunbar, P. Dann*, T. Robinson*, J. Wysolmerski, Yale University, New Haven, CT, NIH, Bethesda, MD.

Male mice lack mammary glands due to the actions of androgens on the mammary mesenchyme during fetal life. The mammary epithelium induces expression of androgen receptor (AR) within the dense mammary mesenchyme and, in response to fetal androgen production, the mesenchyme condenses around the mammary bud, disrupting its stalk and destroying the epithelium. This process fails to take place in PTHrP and PTH/PTHrP receptor knockout embryos. We examined 48 male embryos from matings between heterozygous PTHrP-null mice at E15, a time at which the androgen-mediated destruction of the mammary buds should be well advanced. On whole mount examination, all 10 wild-type male embryos and 27/28 heterozygous PTHrP-null male embryos displayed either no remaining mammary buds, or small remnants of deteriorating buds. In contrast, all 10 homozygous PTHrP-null males demonstrated healthy-appearing mammary epithelial buds, similar in appearance to wild-type female buds. Examination of 10 homozygous receptor-null male embryos revealed the same findings. Histologically, at E15, mammary buds in both PTHrP and receptor knockout embryos lacked the expected mesenchymal condensation; the epithelial stalk was intact, and the buds were indistinguishable from wild-type female buds. Examination by TUNEL assay at E15 revealed massive apoptosis within the condensed mammary mesenchyme and epithelial stalk in wild-type male embryos which was completely lacking in knockout males. Finally, immunohistochemistry revealed that knockout embryos lacked AR expression within the mammary mesenchyme. This was also the case for Tenascin C expression, another marker of differentiated mammary mesenchyme.

In summary, amino-terminal PTHrP and the PTH/PTHrP receptor are necessary for sexual dimorphism during murine mammary gland development. In the absence of PTHrP, androgen receptor fails to be expressed within the mammary mesenchyme and the mammary buds persist. These studies, combined with earlier results localizing PTHrP to the mammary epithelium and the PTH/PTHrP receptor to the mesenchyme, establish that PTHrP is the epithelial signal responsible for induction of AR expression within the dense mammary mesenchyme. In addition, PTHrP is also responsible for the expression of Tenascin C, the other classic marker of mammary mesenchyme. Since the PTH/PTHrP receptor is known to be expressed diffusely within the ventral mesenchyme, it appears that epithelial PTHrP expression serves as a critical signal that regulates cell fate decisions within undifferentiated ventral mesenchyme cells which lead to their differentiation into mammary-specific mesenchyme.

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Stromal Cells are Critical Targets in the Regulation of Mammary Ductal Morphogenesis by PTHrP M. Dunbar, P. Young*, J.P. Zhang*, B. Lanske, J.Orloff, A.Karaplis, G.Cunha*, and J.Wysolmerski.
Yale University, New Haven, Connecticut, University of California at San Francisco, San Francisco, California, Max-Planck-Institut, Martinsried, Germany, McGill University, Montreal, Quebec.

It is known that overexpression or underexpression of PTHrP in the mammary gland leads to severe disruptions in its morphogenesis. Based on these phenotypes it has been suggested that PTHrP participates in the regulation of epithelial-stromal interactions during mammary development. Here we present further data to support such a role. By RNase protection analysis, both PTHrP and the PTH/PTHrP receptor mRNA are expressed in the mammary gland during periods of active ductal morphogenesis. PTHrP mRNA expression is only found within portions of the mammary gland containing epithelial cells, while the PTH/PTHrP receptor mRNA is found within regions of the mammary gland containing epithelium as well as the fat pad alone. By in situ hybridization, PTHrP is expressed in mammary epithelial cells, while the PTH/PTHrP receptor is expressed in the surrounding mesenchyme. The most intense expression of PTHrP and the PTH/PTHrP receptor expression appears to be in the embryonic mammary bud and in terminal end buds during puberty, both sites of active proliferation and ductal morphogenesis during mammary development. Additionally, receptor binding assays demonstrated that mammary stromal cells in culture bind amino terminal PTHrP with a Kd of 8.9 ± 1.4 nM, and respond to amino terminal PTHrP with a four-fold increase in intracellular cAMP levels indicating that these cells have functional PTH/PTHrP receptors. Finally, using heterotypic tissue recombination and transplantation experiments, we have demonstrated that mammary mesenchymal cells must express the PTH/PTHrP receptor in order for these cells to support mammary epithelial cell morphogenesis. Mesenchymal cells taken from PTH/PTHrP receptor-null embryos were not able to support the growth of either receptor-null or wild-type epithelial cells when these tissues were recombined and transplanted under the kidney capsule of recipient mice. However, receptor-null epithelial cells were able to survive and initiate branching morphogenesis when recombined with normal mesenchyme. Together, these results demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal signaling circuit that is necessary for mammary morphogenesis and that stromal cells are a critical targets of PTHrP's effects on mammary morphogenesis.

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THE PTH/PTHrP RECEPTOR IS EXPRESSED IN MAMMARY STROMAL CELLS THROUGHOUT DEVELOPMENT ((Maureen E. Dunbar, James F. McCaughern-Carucci, Jian-Ping Zhang, John Orloff, and John Wysolmerski.)) Yale School of Medicine, New Haven, CT 06520.

Parathyroid hormone-related protein (PTHrP) is a normal product of mammary epithelial cells and recent experiments in our laboratory have demonstrated that over- or underexpression of PTHrP in the murine mammary gland leads to severe disruptions in its development. It is thought that PTHrP may modulate epithelial-stromal interactions that guide mammary growth and development. To study this possibility we have examined the expression of PTHrP and the PTH/PTHrP receptor during various stages of mammary gland development. During both embryonic mammary development as well as post-natal development, by *in situ* hybridization, PTHrP is expressed in epithelial cells, while the PTH/PTHrP receptor is expressed in the surrounding mammary mesenchyme. These data suggest that PTHrP is an epithelial signal that is received by the mammary mesenchyme where it is involved in modulating stromal cell function during mammary development. To test this hypothesis, we have isolated mammary epithelial and stromal cells from mice, and have characterized these cells for PTH/PTHrP receptor expression. RNase protection analysis of RNA from isolated epithelial and stromal cells revealed that mammary stromal cells, but not epithelial cells express the PTH/PTHrP receptor. Receptor binding assays revealed that mammary stromal cells bind amino terminal ^{125}I-PTHrP with a Kd of 8.9±1.4 and the number of receptors on the cell surface was calculated to be 1.26×10^5 receptors/cell. Additionally, treatment of mammary stromal cells in culture with 10^{-7} M PTHrP (1-36) caused a four-fold increase in intracellular cAMP levels indicating that these cells have a functional PTH/PTHrP receptor. To elucidate the mechanisms by which PTHrP regulates mammary development, we have performed subtractive hybridization on RNA from mammary stromal cells treated with or without PTHrP in order to identify genes that are differentially expressed in response to PTHrP. A number of cDNAs have been identified that are differentially expressed in mammary stromal cells in response to PTHrP and experiments are ongoing to identify and clone these genes.

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Parathyroid Hormone-Related Protein: From Hypercalcemia of Malignancy to Developmental Regulatory Molecule

MAUREEN E. DUNBAR, PHD, JOHN J. WYSOLMERSKI, MD,
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ABSTRACT: Parathyroid hormone-related protein (PTHrP) was originally discovered because of its role in humoral hypercalcemia of malignancy (HHM), a common metabolic complication of many types of cancer. In HHM, PTHrP is released into the circulation by malignant cells and cross reacts with parathyroid hormone (PTH) receptors in bone and kidney, which results in hypercalcemia. In recent years, it has become clear that PTHrP is a normal product of many adult and fetal tissues where it appears to act in an autocrine and/or paracrine fashion to regulate organogenesis. This article explores the molecular evolution of PTHrP and how this understanding has begun to shed some light on the molecular mechanisms responsible for the biochemical manifestations of HHM. In addition, the normal biological function of PTHrP is discussed, with an emphasis on its role as a developmental regulatory molecule. KEY INDEXING TERMS: Parathyroid hormone-related protein (PTHrP); Parathyroid hormone (PTH); Humoral hypercalcemia of malignancy (HHM); Organogenesis; Developmental regulatory molecule. [Am J Med Sci 1996;312(6):287–294.]

Parathyroid hormone-related protein (PTHrP) was originally identified as the tumor product responsible for the clinical syndrome of humoral hypercalcemia of malignancy (HHM), a common metabolic complication of many types of cancer.[1–3] Humoral hypercalcemia of malignancy shares many biochemical features with primary hyperparathyroidism, and these similarities are now appreciated at a molecular level. The parathyroid hormone (PTH) and PTHrP genes share a common evolutionary heritage and retain structural similarities that allow both proteins to signal through the PTH receptor (now known as the PTH/PTHrP receptor).[4] Parathyroid hormone is exclusively a product of the parathyroid chief cells, is secreted into the circulation, and acts through PTH/PTHrP receptors in bone and kidney to regulate systemic calcium metabolism after birth.[5] In contrast, PTHrP is produced by a wide variety of cells, is normally excluded from the circulation, and acts through PTH/PTHrP receptors in nonclassic locations to influence cellular growth and differentiation, especially during development.[3] Because these two proteins share a common receptor, when PTHrP gains access to the circulation by virtue of its secretion by a tumor it is able to interact with PTH/PTHrP receptors in the classic locations of bone and kidney to produce the biochemical features of HHM.[4] In this article, we consider the role of PTHrP in HHM and how an understanding of the molecular evolution of PTH, PTHrP and the PTH/PTHrP receptor has brought the understanding of this syndrome full circle. In addition, we also consider the biological function of PTHrP, concentrating on its role as a developmental regulatory molecule.

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Parathyroid Hormone-Related Protein and Humoral Hypercalcemia of Malignancy

Humoral hypercalcemia of malignancy refers to a syndrome of diffuse osteoclastic bone resorption associated with a tumor distant from the skeleton (with or without bone metastasis). It is now recognized as the dominant form of malignancy-associated hypercalcemia (MAHC) causing up to 75% to 80% of consecutive nonselected cases of MAHC occurring in the setting of malignancy.[4,6] Humoral hypercalcemia of malignancy is associated with a distinct subset of tumor types, the most common of which are squamous, renal, and urothelial malignancies. In addition, virtually 100% of hypercalcemic patients with human T-cell leukemia virus (HTLV-

Figure 1. Photomicrograph of a bone biopsy specimen from a patient with humoral hypercalcemia of malignancy. Note the absence of malignant cells, the tremendous increase in osteoclasts and empty lacunae, and the absence of osteoblasts and osteoid. Reprinted with permission from Stewart et al.[42]

1) associated adult T-cell leukemia/lymphoma have HHM.[4] Humoral hypercalcemia of malignancy is characterized biochemically by an elevated serum calcium, a low serum phosphorus, a low PTH level, low 1,25(OH)2 vitamin D levels, and an elevated nephrogenous cAMP (NcAMP) excretion rate.[6] This profile is somewhat similar to that of patients with primary hyperparathyroidism, especially with respect to the NcAMP elevation, which was previously considered a specific test of PTH action because it reflects activation of the renal proximal tubular PTH receptor.[7] However, HHM differs from hyperparathyroidism in terms of its low PTH and 1,25(OH)2D levels and reduced distal tubular calcium resorption.[6] Another characteristic difference is the uncoupling of bone resorption and bone formation in HHM (Figure 1). In HHM, osteoclast activity is increased but bone formation is suppressed, whereas in hyperparathyroidism both bone resorption and bone formation are increased in a coupled fashion.[4]

In the 1960s, the similarities between HHM and hyperparathyroidism led to the suggestion that this syndrome was caused by the secretion of PTH itself by tumor cells and the syndrome was called "pseudohyperparathyroidism."[8] However, by the early 1980s, clinical studies determined that HHM was not caused by ectopic production of PTH but by another factor that seemed able to mimic certain actions of PTH.[6] In fact, the PTH-like nature of this factor with respect to its ability to increase NcAMP production was the key feature that enabled it to be isolated and characterized.[1–3] Investigators from several laboratories used PTH-sensitive renal or bone cAMP assays in vitro to purify the factor from tumors obtained from patients with HHM. This effort required 5 years and a 20,000-fold to 60,000-fold purification and culminated in the discovery of human PTHrP in 1987,[1–3] the cloning of its cDNAs in 1987,[9,10] and the elucidation of the genomic structure for the human PTHrP gene in 1989.[11]

In the near-decade that has elapsed since the initial purification of PTHrP, we have learned much about this peptide and the role in HHM. Parathyroid hormone-related protein is encoded by a complex gene that contains 8 exons spanning 15 kilobases of DNA.[5] Transcription involves the use of at least three distinct promoters, and alternative exon splicing occurs at both the 5′ and 3′ ends of the gene, which leads to a family of mRNA species. These mRNAs all contain AU-rich instability sequences within the 3′ untranslated regions (UTRs) and all have a short half-life—characteristics shared by many lymphokine and protooncogene mRNAs.[5]

Human PTHrP mRNAs encode three different protein isoforms that contain 139, 141, and 173 amino acids, respectively.[5] These isoforms are all identical through amino acid 139 and contain the same prepro sequences.

The first 13 amino acids of PTHrP are highly homologous to the same amino acids of PTH (8 are identical) and, although the peptide regions from amino acids 14–34 differ in primary amino acid sequence, PTHrP and PTH may share similar secondary structure in these regions.[4] As is discussed in greater detail below, it is now known that this homology is not coincidental; the PTHrP and PTH genes share a common ancestry and are members of a small gene family.[5] This information is particularly enlightening regarding the pathogenesis of HHM because these regions of shared primary and secondary structure correspond to the PTH-like regions that are important for receptor binding and activation.[5] Indeed, many studies have revealed that amino-terminal fragments of PTHrP are equipotent with PTH in binding to and activating classical PTH

receptors.[12] Therefore, the majority of the biochemical features of HHM clearly result from tumor secretion of PTHrP into the systemic circulation, with subsequent interaction or cross reaction with PTH/PTHrP receptors located in the skeleton and kidneys.

In the past few years, several lines of evidence confirmed that PTHrP is the causative agent of HHM. First, shortly after cDNAs for PTHrP were cloned, PTHrP mRNA was documented in tumors associated with HHM.[13] Second, synthetic N-terminal fragments of PTHrP have been shown to cause severe hypercalcemia, hypophosphatemia, and elevated NcAMP levels when infused into rodents[14,15] and into human subjects.[16] Third, studies using sensitive immunoassays have revealed elevated levels of circulating PTHrP in patients with HHM.[17] Finally, neutralizing antibodies to PTHrP can reverse hypercalcemia caused by HHM-associated tumors grown in nude mice.[18]

Parathyroid hormone-related protein, therefore, seems to be both necessary and sufficient to explain the key features of HHM. However, certain pathophysiologic features of the syndrome are still not completely understood. These include the uncoupling of bone turnover, the inhibition of 1,25-(OH)2D production, and the apparent reduced potency of PTHrP in increasing distal tubular calcium absorption when compared with PTH.[5,6]

Results of studies in which PTHrP was infused into rodents and/or human subjects have not consistently reproduced these aspects of the syndrome. Because PTHrP appears to cause the biochemical features of HHM by interacting with the same PTH/PTHrP receptors with which PTH interacts to cause hyperparathyroidism, it is difficult to reconcile these differences between HHM and hyperparathyroidism.

Reviews should be consulted for a thorough discussion of these issues,[4,19] but here are several brief potential explanations: 1) most studies have used short amino-terminal fragments of PTHrP in the designs, but other regions of the molecule may be important with respect to these differences, and the full spectrum of circulating forms of PTHrP has yet to be determined in patients with HHM; 2) tumors may cosecrete other factors that inhibit bone formation and/or 1,25-(OH)2 D synthesis; or 3) coexistent debilitation and/or the severity of the hypercalcemia may suppress bone formation and 1,25 (OH)2 D formation in these patients independent of the direct effects of PTHrP.[4] These points remain areas of active investigation and are of some importance, given the reproducibility of the biochemical and clinical phenotype in patients with HHM.

The scope of this review does not extend to discussion of the normal physiologic regulation of PTHrP production, but we wish to establish two points before continuing. First, the PTHrP gene is expressed in a wide variety of normal fetal and adult tissues, where it is thought to function in an autocrine or paracrine fashion.[5] These sites include the tissues of origin of most, if not all, HHM-associated tumors. Second, with the possible exception of tumors of neuroendocrine origin, PTHrP is principally secreted in a constitutive fashion and, in contrast to many other hormones, is not usually stored.[5] Therefore, secretion is controlled at the level of PTHrP production, not at the level of its release. For these reasons, the current study of the molecular mechanisms responsible for HHM focused on the mechanisms responsible for PTHrP gene expression in tumor cells. In addition, because PTHrP is a natural product of the tissues of origin of these tumors, the current feeling is that the mechanisms of PTHrP gene expression in tumors will probably represent dysregulation of existing physiologic controls as opposed to mechanisms of true ectopic expression (such as gene duplication or rearrangement).

Study of the regulation of PTHrP gene expression in malignancy is an emerging field, but there are several systems that have begun to yield useful results. As was mentioned previously, HHM is a common complication of HTLV-1–associated adult T-cell leukemia/lymphoma. Normal T-cells do not express the PTHrP gene, but virtually 100% of those infected with HTLV-1 do. In this syndrome, a viral protein (Tax) seems to act as a transactivating factor that stimulates PTHrP gene transcription.[20] These effects appear to be mediated by specific Sp1 and Ets transcription factor binding sites located within the PTHrP gene promoter region, but the exact mechanisms by which Tax interacts with these factors to activate PTHrP gene expression remains unknown.[21]

Our laboratory focused on the control of PTHrP gene expression in renal and squamous carcinomas. Renal carcinomas appear to fall into one of two groups, those that express the PTHrP gene and those that do not. All of the PTHrP-expressing cell lines tested to date cause hypercalcemia when grown in athymic mice, but negative-expressing lines have no such effect.[22] The ability of these lines to express the PTHrP gene closely correlates with the methylation status of specific nucleotides (CpG residues) within the promoter region of the gene.[23] When these nucleotides are methylated, no transcription of the gene occurs, but when they are unmethylated, the cells transcribe the gene at a high level. Therefore, in renal cancers, specific biochemical modifications of the DNA that encodes the PTHrP gene appear to act as a switch that determines whether the transformed cells will express the gene and cause HHM. How this "switch" is controlled during cell transformation and tumor growth remains an open area of investigation.

Unlike the "on/off" situation in renal carcinomas,

PTHrP gene expression in squamous carcinomas appears to be ubiquitous. Nevertheless, squamous carcinomas exhibit a wide spectrum of expression levels and, in an individual cell line, the level of expression correlates well with the ability to cause hypercalcemia when grown in athymic mice.[24] The level of expression in squamous lines studied thus far also differs from that of renal carcinoma cells in that it appears not to be set by cis-modification of the DNA itself but rather by the differential activity of trans-activating transcription factors.[24] In addition, recent studies have implicated the tumor suppressor gene p53 as a possible negative modulator of PTHrP expression in squamous tumor cells.[25] Although the identity of the specific factors that control PTHrP expression in squamous tumors remains an area of active investigation, the work cited above suggests that the level of PTHrP expression in a given tumor is the sum of specific positive and negative regulatory factors and that the particular mix of these factors will ultimately dictate if a particular tumor causes HHM.

As these three examples demonstrate, studies that examined the regulation of PTHrP gene expression in tumors failed to identify any singular mechanism that can explain HHM at a molecular level. These early results most likely reflect the complexity of the gene and the normal transcriptional regulation, as well as the diversity of pathways to the transformed phenotype. Although certain paradigms may emerge, the specific molecular events that determine cancer-related PTHrP production will most likely prove to be tumor-type specific.

Parathyroid Hormone-Related Protein and Parathyroid Hormone: A Gene Family

The amino-terminal portion of PTHrP and PTH are highly homologous, sharing 8 of the first 13 amino acids.[5] This knowledge generated a great deal of excitement when PTHrP was first isolated because, as discussed above, it provided a rationale for the similarities between hyperparathyroidism and HHM. With the cloning of the PTHrP gene, it became clear that the homologous amino-terminal regions of PTH and PTHrP were the results of a true evolutionary relationship between the PTH and PTHrP genes. The human PTHrP gene is located on the short arm of chromosome 12, whereas the human PTH gene is located on the short arm of chromosome 11; these two chromosomes arose as the result of an ancient tetraploidization event.[9,16] In addition, there is a similar organization to the PTH gene and the portions of the PTHrP gene that encode the prepro and coding regions of PTHrP.[5] These facts suggest that PTH and PTHrP arose from a common ancestral gene as a result of this tetraploidization event, some 200 to 300 million years ago.[26]

This time frame is consistent with the apparent emergence of PTH as a distinct gene in amphibians.[27] (Although there have been several preliminary reports of PTH-like sequences in fish, no distinct gene has been cloned.) The PTHrP gene has been characterized as far back as chickens and appears to be highly conserved over evolutionary time, but there is currently no information as to the presence of a distinct PTHrP gene in either amphibians or fish.[5] Whatever the exact point of divergence from the ancestral gene, it is clear that these two genes evolved separately. The PTH gene has a simple organization; it is expressed solely by the parathyroid chief cells and PTH functions as a classic systemic hormone to regulate systemic calcium homeostasis. In contrast, the PTHrP gene has a complex organization; it is expressed in a wide variety of cells and PTHrP appears to function to influence cellular growth and differentiation in an autocrine and/or paracrine fashion.

The cloning of the PTH receptor in 1991[28] provided additional evidence of the relationship between PTH and PTHrP. It had been known that amino-terminal fragments of PTH and PTHrP were equally potent in ability to bind and activate this receptor,[12] but the finding that the receptor is expressed in many tissues other than the classic PTH-target tissues suggested that the ability of both ligands to signal via this receptor was no accident and that this molecule subserved the physiologic functions of both ligands.[28] Correspondingly, it was designated the PTH/PTHrP receptor.

That the PTH/PTHrP receptor is involved in the transduction of PTHrPs biological effects as well as those of PTH is most evident during development when PTHrP and the PTH/PTHrP receptor genes are expressed coordinately in adjacent cells beginning from the late morula stage onward (see below).[29,30] The PTH/PTHrP receptor also appears to be well conserved functionally, as a recent preliminary report demonstrated its existence in amphibians as well as the ability of the amphibian receptor to respond to human PTH and PTHrP.[31] It is interesting to speculate as to whether the PTH/PTHrP receptor will function in lower species to subserve the functions of the ancestor to PTH and PTHrP and, in that respect, it will be of great interest to see the point at which the receptor gene emerged in evolution.

What has emerged from this information is a picture of PTH and PTHrP as two ancestrally related ligands that share a common receptor. The systemic calcium metabolism-regulating functions of PTH are governed by PTH/PTHrP receptors in the classic target tissues of bone and kidney, and the functions of PTHrP are governed by PTH/PTHrP receptors on a variety of different cell types located in tissues not participating in systemic calcium metabolism. Obviously, if this is the case, there must be mechanisms to ensure that the correct ligand talks to the correct

receptor population. Although the details of these mechanisms remain the focus of current investigations, they almost certainly involve differences in the density of receptors in different locations (higher in PTH-target tissues),[5] the temporal and spatial regulation of ligand and receptor expression (adjacent expression of PTHrP and PTH/PTHrP receptor expression during development),[30] and the compartmentalization of the two ligands (PTH in the circulation and PTHrP in local microenvironments).[5] With these data in mind, it becomes clear that HHM represents the consequences of a breakdown of the barriers that normally separate the realms of PTH and PTHrP. Through a combination of overproducing PTHrP and disrupting the barriers that normally exclude PTHrP from the circulation, tumors allow PTHrP to gain access to PTH/PTHrP receptors in bone and kidney that are normally reserved for its cousin, PTH.

Parathyroid Hormone-Related Protein as a Developmental Regulatory Molecule

Soon after the discovery of PTHrP, it became clear that PTHrP was expressed during embryogenesis in a wide variety of tissues derived from all three germ layers.[5] Recent studies have found a similar widespread distribution of PTH/PTHrP receptor expression during fetal life.[30] In fact, PTHrP and the PTH/PTHrP receptor have been reported to be one of the earliest peptide hormone/receptor pairs to be detected during development in mice as they are expressed from the late morula stage onward.[29] Work by Lee et al has demonstrated that, in the developing rat embryo, PTHrP is predominantly expressed in surface epithelia, such as skin and respiratory epithelia, whereas the PTH/PTHrP receptor is predominantly expressed in stromal cells immediately adjacent to the PTHrP-expressing epithelial cells.[30] This type of "hand-in-glove" pattern of expression suggests that PTHrP functions as a paracrine factor and may be involved in epithelial–mesenchymal interactions, a process important in the development of many epithelial organs. In fact, several recent experiments in transgenic mice demonstrated that over- or underexpression of PTHrP has profound effects on the development of several organs.

Parathyroid Hormone-Related Protein in Developing Bone. Two recent in vivo studies suggest that PTHrP regulates the rate of programmed chondrocyte differentiation in developing endochondral bone. First, Karapalis et al disrupted the PTHrP gene in mice by homologous recombination, producing mice that suffered from a form of chondrodysplasia that resulted in death shortly after birth.[32] These mice displayed short-legged dwarfism and had premature and inappropriate ossification of developing skeletons. Histologically, there was a disruption in the normal architecture of the growth plate, with a pronounced decrease in the numbers of resting and proliferative chondrocytes, as well as the aforementioned premature endochondral/perichondral ossification. These findings suggested that there was an acceleration in the chondrocyte developmental program. Second, targeted overexpression of PTHrP to proliferative chondrocytes using the collagen II (col II) promoter in transgenic mice also led to severe skeletal abnormalities.[33] Characterization of these mice revealed that they have a profound delay in the program of chondrocyte differentiation as well as a failure in the ability to ossify the skeleton—the mirror image of the findings of the PTHrP-null mice. Together, these findings implicate PTHrP as an important regulator of the developmental program that controls the orderly sequence of events associated with epiphyseal growth and cartilaginous mineralization.

Results of studies that employed immunohistochemistry and in situ hybridization have demonstrated PTHrP expression in immature chondrocytes in the mesenchyme of developing limbs and later in perichondrocytes and in maturing and hypertrophic chondrocytes of the fetal growth plate. The PTH/PTHrP receptor has been localized to prehypertrophic and perhaps proliferative chondrocytes within the growth plate.[34,35] These are appropriate locations for both ligand and receptor if, as suggested by the in vivo experiments cited above, PTHrP acts as a negative regulator of chondrocyte differentiation. In fact, this interpretation is supported by a series of older experiments in vitro that have shown that PTHrP and PTH are able to retard chondrocyte differentiation when added to cartilage cell culture systems.[36] Finally, it has recently been found that a rare type of human chondrodysplasia, called Jansen-type metaphyseal chondrodysplasia, is caused by activating mutations in the PTH/PTHrP receptor.[37] These individuals have "pseudo-growth plates" consisting of masses of retained cartilage, reminiscent of the histology seen in the col II-PTHrP overexpressing mice. Therefore, it would appear that PTHrP acts as a break on chondrocyte differentiation and that there may be a gradient of PTHrP concentration within the developing growth plate important to maintaining the orderly sequence of chondrocyte proliferation and differentiation that permits linear growth of the skeleton.

Parathyroid Hormone-Related Protein Regulates Epithelial-Mesenchymal Interactions in Skin and Mammary Glands. Normal human keratinocytes were the first nonmalignant cells shown to secrete PTHrP,[5] and it is now known that PTHrP is found throughout the epidermis at as early as 14 days of gestation in the rat.[5] To investigate the function of PTHrP in skin, we developed a transgenic model using the keratin 14 (K14) promoter to target PTHrP overexpression to basal keratinocytes and outer

root sheath cells of hair follicles—sites that normally express PTHrP at lower levels.[38] Overexpression of PTHrP in this manner resulted in a profound delay or a complete failure of hair follicle development. The development of hair follicles is critically dependent on the proper exchange of signals between fetal keratinocytes and dermal mesenchymal cells. It is also known that PTHrP expression is limited to epithelial cells and PTH/PTHrP expression is limited to the surrounding mesenchymal cells in developing hair follicles.[30] In addition, it has been demonstrated that PTHrP stimulates adenylyl cyclase activity in dermal fibroblasts and that dermal fibroblasts produce a soluble factor(s) that stimulates PTHrP production in squamous carcinoma cells.[5] Therefore, it appears that there is a paracrine loop in skin in which the epidermis produces PTHrP, which modulates the ability of the fetal dermis to support hair follicle development. In turn, it appears that dermal cells may regulate the production of PTHrP in keratinocytes. Although the details remain to be sorted out, it is likely that PTHrP exerts its influence on hair follicle development through such a paracrine loop.

The mammary gland is another epithelial organ that is dependent on epithelial-mesenchymal interactions for its proper growth, which occurs through a process known as branching morphogenesis. It is known that PTHrP is produced by both luminal epithelial cells and myoepithelial cells of the mammary gland.[39,40] In addition, it is known that myoepithelial cells express the PTH/PTHrP receptor,[40] and preliminary studies in this laboratory have demonstrated that mammary stromal cells do as well. Therefore, as in hair follicle development, PTHrP and the receptor are appropriately positioned to participate in the regulation of epithelial–mesenchymal crosstalk in mammary development. This laboratory has recently reported that overexpression of PTHrP in the myoepithelial cells of transgenic mice results in abnormal mammary gland development (Figure 2).[41] In these mice, PTHrP appears to inhibit the overall rate of ductal proliferation and elongation and to impair branching morphogenesis during mammary gland development. The nature of this phenotype suggests that PTHrP is an important regulator of epithelial–mesenchymal crosstalk during mammary gland development. The working hypothesis is that PTHrP, produced by mammary epithelial cells, acts on mammary stromal cells and modulates the ability to support mammary gland development.

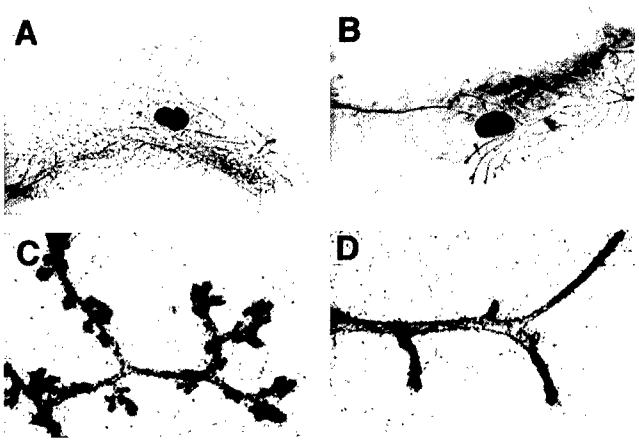

Figure 2. Whole mount analysis of mammary glands from 6-week-old PTHrP-overexpressing mice. Mammary glands from 6-week-old transgenic mice (B and D) and a control littermate (A and C) were resected in toto and stained with carmine aluminum. A and B represent low-power views of whole mounts of the #4 inguinal mammary gland from normal (A) and transgenic (B) mice. C and D represent high-power views of the same samples. Note the overall retarded growth of the ducts into the fat pad of the transgenic gland (B). Also, note the simpler ductal architecture with many fewer branches in transgenic (B) as compared with normal (B) glands, as well as the reduction of terminal branches in transgenic glands (D). Reprinted with permission from Wysolmerski et al.[41]

Conclusion

Parathyroid hormone-related protein was discovered because of the role as the causative agent of the syndrome of humoral hypercalcemia of malignancy. During the past decade, it has become clear that this protein is widely expressed in normal tissues, where it serves as a local autocrine and/or paracrine factor involved in the regulation of cellular growth and differentiation. In addition, it is now understood that the PTHrP and PTH genes are related and that they retained the mutual use of a common receptor, the PTH/PTHrP receptor. Therefore, it is now recognized that HHM is the result of the dysregulation of PTHrP production by malignant cells in such a manner that PTHrP gains access to the circulation (from which it is normally excluded) and interacts with a PTH/PTHrP receptor pool normally reserved exclusively for PTH.

In the past 3 or 4 years, transgenic technology has led to enormous strides in the understanding of the functional role(s) of PTHrP, which clearly implicates the protein as a developmental regulatory molecule. The growth of hair follicles and mammary gland development are both examples of "inductive development," in which there is a rich exchange of information between epithelial cells and neighboring stromal cells. Although bone is not a classic epithelial organ, development is also critically dependent on the proper exchange of information between neighboring chondrocytes, stromal cells, and marrow cells, and the program of endochondral bone formation is fundamentally similar to the program of keratinocyte differentiation leading to the development of the

mature hair follicle. Therefore, the developmental phenotypes of the transgenic mice discussed all suggest that PTHrP may be an important participant in the regulation of cell-cell interactions during organogenesis.

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Parathyroid Hormone-Related Protein: A Developmental Regulatory Molecule Necessary for Mammary Gland Development

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Parathyroid hormone-related protein (PTHrP) was originally identified as the tumor factor responsible for a clinical syndrome known as humoral hypercalcemia of malignancy. It is now appreciated that PTHrP[3] is a developmental regulatory molecule expressed during the formation of a wide variety of organs. Recently, our laboratory has demonstrated that PTHrP is necessary for mammary gland development. Our studies have suggested that this molecule participates in the regulation of epithelial-mesenchymal interactions during embryonic mammary development and perhaps also during adolescent ductal morphogenesis. In addition, it has been suggested that PTHrP plays a critical role in the establishment of bone metastases in breast cancer. In this article, we will discuss the current knowledge of the mechanisms underlying PTHrPs actions during normal mammary development and in breast cancer.

KEY WORDS: Epithelial-mesenchymal interactions; branching morphogenesis; PTH/PTHrP receptor; mammary mesenchyme.

INTRODUCTION

Parathyroid hormone-related protein or PTHrP was originally discovered as a tumor product responsible for the clinical syndrome of humoral hypercalcemia of malignancy (HHM), a common metabolic complication of many types of cancer (1). It is now appreciated that PTHrP is the product of a wide variety of normal tissues and that its capacity to produce HHM is the result of an intriguing evolutionary relationship with parathyroid hormone (2). Furthermore, recent data have demonstrated that PTHrP plays important roles in the development of several organs, including the mammary gland (3–5). In this review, we will discuss the current knowledge of the roles that PTHrP plays during mammary development and in breast cancer. There are now several indepth reviews of PTHrP available, and the reader is referred to these for a detailed discussion of PTHrP and its gene (2–5). However, before proceeding, we will briefly touch upon several themes of PTHrP biology that will serve as necessary background for a full understanding of the actions of PTHrP in the mammary gland.

HUMORAL HYPERCALCEMIA OF MALIGNANCY—THE ISOLATION AND CHARACTERIZATION OF PTHrP

Humoral hypercalcemia of malignancy refers to a syndrome of diffuse osteoclastic bone resorption associated with a tumor distant from the skeleton. Patients suffering from this syndrome have a biochemical profile of hypercalcemia and hypophosphatemia similar to that seen in primary hyperparathyroidism.

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[3] **Abbreviations:** PTHrP, parathyroid hormone-related protein; PTH, parathyroid hormone; HHM, humoral hypercalcemia of malignancy; BMP, bone morphogenetic protein; K14, keratin 14; ECM, extracellular matrix; TGF-β, transforming growth factor β.

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In fact, historically it was hypothesized that the tumors associated with this syndrome might be making parathyroid hormone (PTH) in an ectopic fashion. However, studies performed in the early 1980s suggested that HHM was caused by a circulating substance that was clearly distinct from PTH, although it had similar actions on PTH-target organs such as bone and kidney (6). These observations laid the groundwork for the purification of PTHrP from human tumors or cell lines associated with HHM (7–9), and were followed rapidly by the isolation of PTHrP cDNAs (10, 11) and the elucidation of the genomic structure of the human PTHrP gene in the late 1980s (12–14).

The human PTHrP gene is a complex transcriptional unit containing 8 exons and spanning 15 kilobases of DNA (2). The PTHrP genes of several other species have also been characterized, and it appears that the majority of the PTHrP coding region has been highly conserved throughout evolution. In all species, the first 13 amino acids of PTHrP are highly homologous to the same amino acids of PTH (8 are identical) and, although the peptide regions from amino-acids 14–34 differ in their primary amino acid sequence, PTHrP and PTH appear to share similar secondary structure in these regions (21). As will be discussed in greater detail later, this homology reflects the fact that the PTHrP and PTH genes share a common ancestry and are members of a small gene family. This information was particularly enlightening in regard to the pathogenesis of HHM, since these regions of shared primary and secondary structure correspond to the PTH-like regions that are important for receptor binding and activation. Indeed, many studies have now documented that amino-terminal fragments of PTHrP and PTH share the use of the same receptor, termed the PTH/PTHrP receptor (discussed later).

Another interesting aspect of PTHrP biology is the fact that the primary transcript of PTHrP is a polyprotein, similar to pro-opiomelanocortin (POMC) (2). The amino acid sequence of PTHrP contains multiple clusters of basic amino acids arranged in pairs, triplets and quadruplets that serve as post-translational processing sites. As a consequence, there are multiple forms of PTHrP, including several species containing the "PTH-like" amino-terminus, as well as mid-region and C-terminal fragments, all of which appear to have distinct biological actions subserved by distinct receptors. The study of the post-translational processing of PTHrP is ongoing, but it is clear that, similar to the case of POMC, different tissues expressing the PTHrP gene may generate different peptides with distinct biological profiles (2,4,5).

Amino-terminal fragments of PTHrP make use of a G protein-coupled, 7-transmembrane-spanning receptor, termed the PTH/PTHrP receptor. This receptor was cloned in 1991, and it is the "classical" PTH receptor involved in systemic calcium homeostasis (15). Prior to its isolation, several groups had shown that amino-terminal fragments of PTH and PTHrP were equipotent in stimulating this receptor (16). However, once its expression pattern was examined, it became clear that this protein also serves as a PTHrP receptor, and it was subsequently named the PTH/PTHrP receptor. Like PTHrP, the PTH/PTHrP receptor is widely expressed and is found in many tissues that are not involved in the regulation of calcium homeostasis. Furthermore, in these sites it is often expressed in cells adjacent to those cells expressing PTHrP (17). Although it is now clear that amino-terminal PTHrP acts through the PTH/PTHrP receptor, the receptors that recognize other fragments of PTHrP remain uncharacterized. There appear to be receptors for the mid-region fragment of PTHrP, and there may be other receptors that recognize the amino-terminal portions of PTHrP as well (18–20). The characterization and isolation of these other PTHrP receptors remains an area of active research.

PTH and PTHrP: A Gene Family

Recognition of the homology between the amino-terminal portions of PTHrP and PTH generated a great deal of excitement when PTHrP was first isolated because it provided a rationale for the similarities between hyperparathyroidism and HHM. With the cloning of the PTHrP gene it became clear that the homologous amino-terminal regions of PTH and PTHrP were the result of a true evolutionary relationship between the PTH and PTHrP genes. The human PTHrP gene is located on the short arm of chromosome 12, while the human PTH gene is located on the short arm of chromosome 11 (11); these two chromosomes arose as the result of an ancient tetraploidization event (21). In addition, there is a similar organization to the PTH gene and the portions of the PTHrP gene encoding the prepro and coding regions of PTHrP (12–14). These facts suggest that PTH and PTHrP arose from a common ancestral gene some 200 to 300 million years ago, a time frame consistent with the apparent emergence of PTH as a distinct gene in amphibians

(22). Although there have been several preliminary reports of PTH-like sequences in fishes, no distinct gene has been cloned. The PTHrP gene has been characterized as far back as chickens and appears to be highly conserved over evolutionary time, but there is currently no information as to the presence of a distinct PTHrP gene in either amphibians or fishes. Whatever the exact point of divergence from their ancestral gene, it is clear that these two genes have evolved separately. The PTH gene has a simple organization; it is expressed solely by the parathyroid chief cells, and PTH functions as a classical peptide hormone to regulate systemic calcium homeostasis. In contrast, the PTHrP gene has a complex organization; it is expressed in a wide variety of cells, and PTHrP appears to function to influence cellular growth and differentiation in an autocrine and/or paracrine fashion. What has emerged from this information is a picture of PTH and PTHrP as two ancestrally-related ligands that share a common receptor. The systemic calcium metabolism-regulating functions of PTH are mediated by PTH/PTHrP receptors in the classical target tissues of bone and kidney, and the functions of PTHrP are mediated by PTH/PTHrP receptors on a variety of different cell types located in tissues not participating in systemic calcium metabolism (16).

PTHrP as a Developmental Regulatory Molecule

Soon after its discovery, it became clear that PTHrP was expressed during embryogenesis in a wide variety of tissues derived from all three germ layers (4). Recent studies have found a similar widespread distribution of PTH/PTHrP receptor expression during fetal life (17). In fact, PTHrP and the PTH/PTHrP receptor have been reported to be one of the earliest peptide hormone/receptor pairs to be detected during mouse development, being expressed from the late morula stage onward (23,24). The exact role that PTHrP plays during early development is still unclear, but recent studies have documented that PTHrP has important functions during organogenesis.

At this point, there are two well documented areas in which PTHrP appears to play important roles. The first is the participation of PTHrP in fetal bone development. Here, PTHrP has been shown to regulate the rate of chondrocyte differentiation within the growth plate of the developing long bones (25–28) where it appears to act downstream of Indian hedgehog and BMP's (29–31). The second area in which PTHrP appears to act during organogenesis is in the regulation of epithelial-mesenchymal interactions during the formation of epithelial organs. In this regard, PTHrP has been implicated in the regulation of the development of hair follicles (32), teeth (33), the lungs (34, William Philbrick, unpublished observations), and the mammary gland (35). To date, we have the most detailed information about the effects of PTHrP on mammary development, and we will now turn our attention to this topic.

The Role of PTHrP in Embryonic Mammary Gland Development

Recent studies from our laboratory have documented that PTHrP is necessary for mammary development (35). We had been interested in studying the effects of the absence of PTHrP on mammary development, but were faced with a dilemma. Much of mammary development occurs postnatally, but PTHrP-knockout mice die at birth due to skeletal abnormalities. In order to circumvent this problem, we devised a rescue strategy that reintroduced PTHrP back into the developing bones of the PTHrP-knockout mouse. The resulting mouse was devoid of PTHrP in all tissues except for the skeleton. This strategy was successful in correcting the bony abnormalities caused by disruption of the PTHrP gene and the "rescued-knockout" mice survived past birth, allowing us to observe the post-natal consequences of the lack of PTHrP on tissues other than the skeleton (33,35). These mice had multiple abnormalities in ectodermally-derived structures such as the epidermis, teeth, sebaceous glands and mammary glands that were reminiscent of a group of human disorders known as ectodermal dysplasias (36). The effects on the mammary gland were the most dramatic. As demonstrated in Fig. 1, these mice had a well-formed mammary fat pad but lacked any mammary epithelial structures whatsoever. In addition, they did not have nipples, which suggested to us that in the absence of PTHrP mammary development fails during embryonic life. We therefore returned to the original PTHrP-knockout model, and found that the failure of mammary development seen in the "rescued-knockout" mice appears to result from a disregulation of epithelial/mesenchymal interactions; in the absence of PTHrP, the mesenchyme is functionally incompetent and cannot support proper epithelial development.

Before discussing the mammary phenotype of PTHrP-knockout mice in detail, it is useful to review

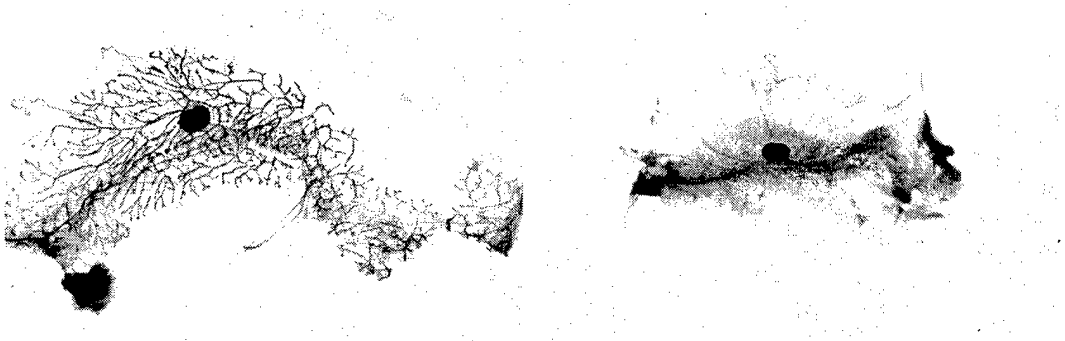

Fig. 1. Whole mount analysis of mammary glands from PTHrP-null mice and normal littermates. The fourth inguinal mammary glands were resected from 4-month-old normal and PTHrP-null mice, fixed in acid ethanol and stained with carmine aluminum. The normal gland (left) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In contrast, the PTHrP-null gland (right) is devoid of epithelial structures; only the lymph node and vasculature are present within the fat pad. [Reprinted with permission from (33).]

the embryonic development of the murine mammary gland (37, 38) (see (39) for a more detailed description). This is essentially a two-step process. The first step involves the formation of five pairs of mammary buds. These structures form as the result of an invagination of epidermal cells at ten characteristic locations along an imaginary line (sometimes referred to as the mammary or milk line) stretching between anterior and posterior limb buds bilaterally. The mammary buds first become visible on E10 and are fully formed by E12. On E13, the fetal testes begin to produce androgen, and in male embryos of most strains of mice, this leads to the destruction of the mammary buds. In response to androgens, the mammary mesenchyme condenses around the neck of the mammary bud and by E14, severs its connection to the epidermis. In most male mice, the remaining mammary epithelial cells subsequently degenerate by birth. In female mice, the mammary buds remain quiescent until E16 when they undergo a transition into the second step of embryonic development, the formation of the rudimentary ductal tree. This process involves the elongation of the mammary bud, its penetration into the mammary fat pad precursor and the initiation of ductal branching morphogenesis. By birth, this process gives rise to an epithelial tree consisting of 15–20 branching ducts contained within the mammary fat pad. This initial pattern persists until puberty, at which time the mature virgin gland is formed through a second round of branching morphogenesis, regulated by circulating hormones (discussed below).

In the absence of PTHrP, there are two major defects in embryonic mammary development. The first is a loss of the normal sexual dimorphism. In male PTHrP-knockout embryos the mammary buds do not degenerate at E14–E15. There is a complete lack of the androgen-mediated mesenchymal condensation and the neck of the mammary bud is well preserved, so that the mammary buds in male and female PTHrP-knockout embryos are indistinguishable and resemble those in wild-type female embryos at E15 (Dunbar and Wysolmerski, unpublished observations). These results are similar to those seen in mice with testicular feminization, or in embryos in which the fetal testes have been ablated (40,41), suggesting to us that in PTHrP knockout embryos, there was some failure of the mesenchymal androgen response. In fact, preliminary studies in our laboratory have demonstrated that PTHrP is necessary for androgen receptor expression in the dense mammary mesenchyme (Dunbar and Wysolmerski, unpublished observations).

The second major defect in these mice is a complete failure of mammary epithelial development at the transition between the two phases of development outlined earlier (35). In PTHrP-knockout mice, mammary buds form normally. However, between E16 and E18, they fail to undergo the initial round of ductal branching morphogenesis (Fig. 2). Instead, in PTHrP-knockout embryos, the mammary rudiment remains bud-like in appearance, does not penetrate into the fat pad precursor, and becomes surrounded by a dense condensation of stroma. Subsequently, the mammary epithelial cells degenerate and, by birth, disappear altogether. At this point, it is not clear how the epithelial cells die. Our studies to date have supported neither necrosis nor classical apoptosis as a mechanism (Fig. 3). Nonetheless, these findings explain the lack of an epithelial duct system in the "rescued-knockout" mice,

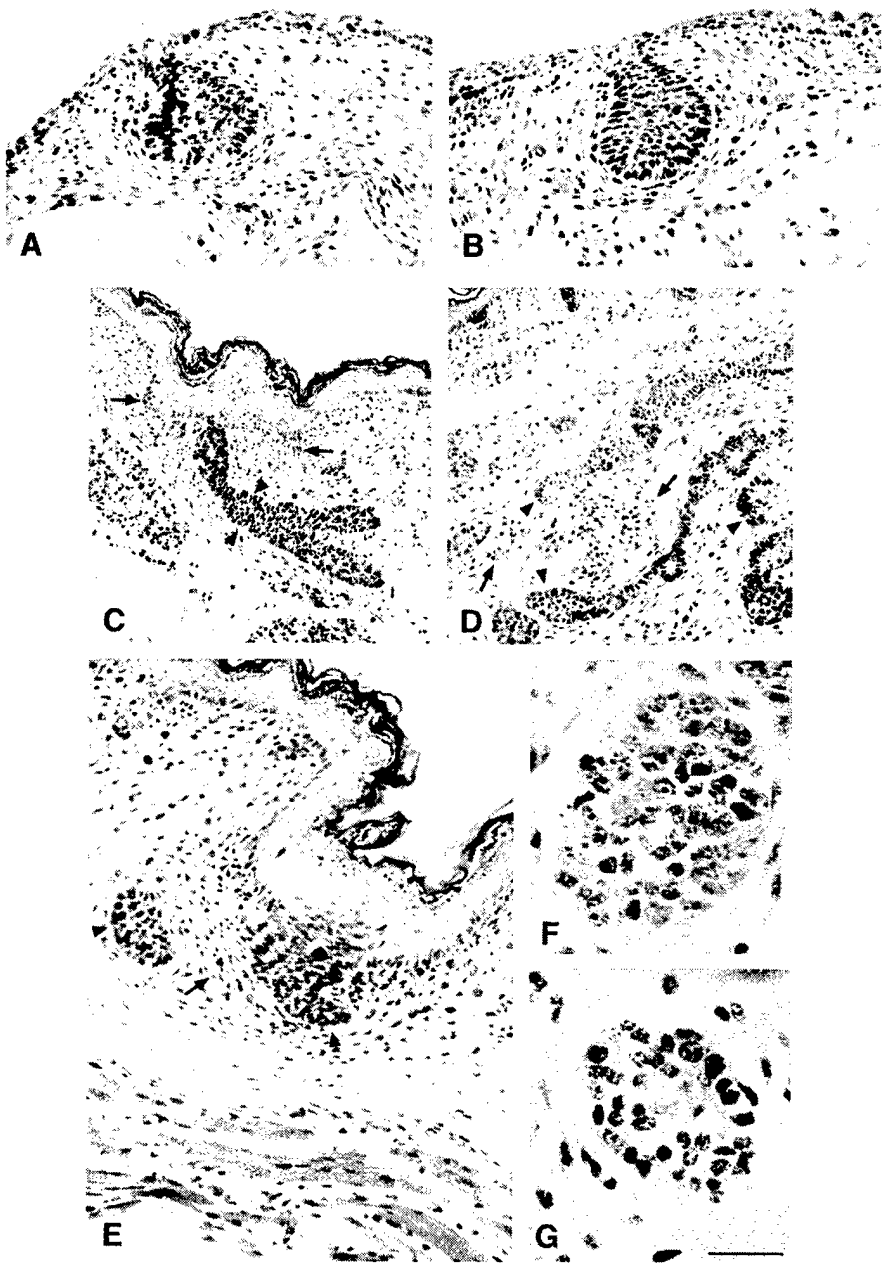

Fig. 2. Histologic comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. A and B are photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15. At this stage, the mammary bud consists of an invagination of mammary epithelial cells surrounded by a condensation of mammary mesenchyme, and the microscopic appearance of the PTHrP-knockout buds was entirely normal. (C)–(G) are photomicrographs of H and E-stained sections through mammary glands dissected from PTHrP-knockout (E and G) and normal littermate (C, D, and F) embryos at E18. In a normal embryo (C and D) one can see the primary epithelial duct (arrowhead in C) arising from the epidermis and extending below the dermis where it branches (arrowheads in D) and makes contact with the preadipocytes (arrows in D) within the developing fat pad. In contrast, in the PTHrP-knockout embryos (E) the epithelial duct (arrowheads) does not extend out of the upper regions of the dermis and becomes surrounded by an abnormally dense condensation of fibroconnective tissue (arrow in E). (F) and (G) are high power photomicrographs of mammary epithelial ducts in crossection taken from a normal (F) and PTHrP-knockout (G) embryo at E18. Note that in the knockout duct (G) the epithelial cells appear to be degenerating; many nuclei are pycnotic, the cell cytoplasm appears reduced and somewhat vacuolated and the cells are separating from the basement membrane. Scale bar represents 16 microns in (A) and (B), 25 microns in (C) and (D), 17 microns in E, and 5 microns in (F) and (G). [Reprinted with permission from (33).]

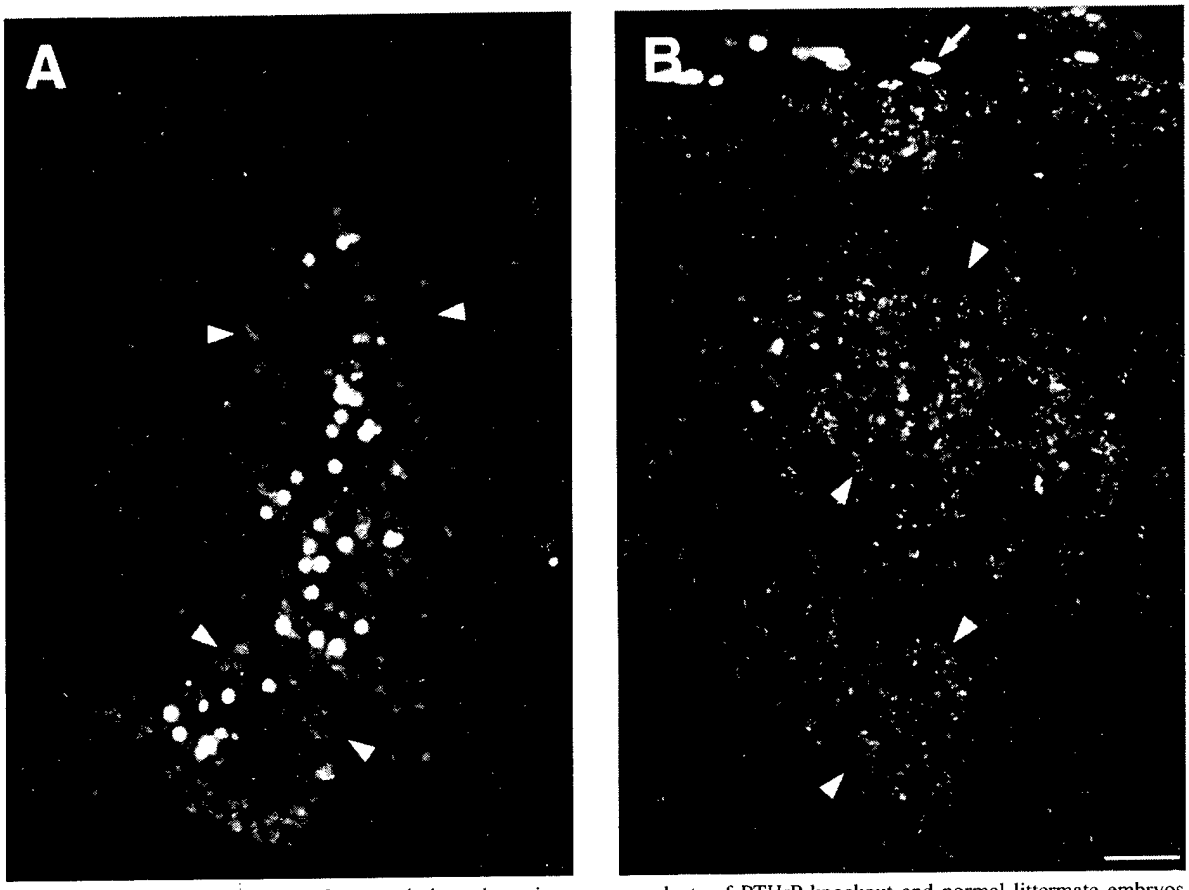

Fig. 3. Histologic determination of apoptosis in embryonic mammary ducts of PTHrP-knockout and normal littermate embryos. TUNEL assay performed on sections of mammary ducts taken from normal (A) and PTHrP-knockout embryos (B) at E18. Note the large number of apoptotic cells (bright nuclei) in the central portion of a normal duct (borders outlined by arrowheads) in (A). In contrast, there are no positively-staining cells in the knockout ducts (outlined by arrowheads) in (B). Despite the lack of positive cells in the mammary ducts in the knockout embryos, cells in the upper layers of the epidermis continued to stain (see arrow), as was the case in normal embryos (not shown). Scale bar represents 17 microns.

and it would appear that PTHrP is necessary for ductal outgrowth and/or epithelial cell survival. In support of this hypothesis we also found that reintroduction of PTHrP into the epithelial cells of the PTHrP knockout mammary buds at E15–E16 by breeding a keratin 14-PTHrP transgene onto the homozygous PTHrP-null background results in the restoration of near-normal embryonic mammary development (35).

As described above, PTHrP is a polyprotein and gives rise to several biologically-active peptides (4–6). We suspected that the mammary phenotype of the PTHrP knockout embryos was a consequence of the lack of amino-terminal PTHrP because the effects of PTHrP-overexpression on ductal morphogenesis were due to this portion of the molecule (discussed below). In order to test this hypothesis, we examined the effects of the ablation of the PTH/PTHrP receptor gene on embryonic mammary development. These embryos phenocopy the PTHrP knockout mice. There is a loss of the expected sexual dimorphism at E14–E15 and the mammary buds degenerate at E16–E18, a pattern that is identical to that seen in PTHrP knockouts (35). Hence, amino-terminal PTHrP must interact with the PTH/PTHrP receptor in order for mammary epithelial development to proceed past the mammary bud stage.

In order to begin to understand the mechanisms by which PTHrP might act during embryonic mammary development, we next determined the cellular localization of PTHrP and PTH/PTHrP receptor expression by in situ hybridization (35,42). PTHrP mRNA is expressed at high levels in mammary epithelial cells of the embryonic mammary bud from E12 on, as well as in the growing ducts during the initial phases of ductal branching morphogenesis at E18 (Fig. 4A–4C).

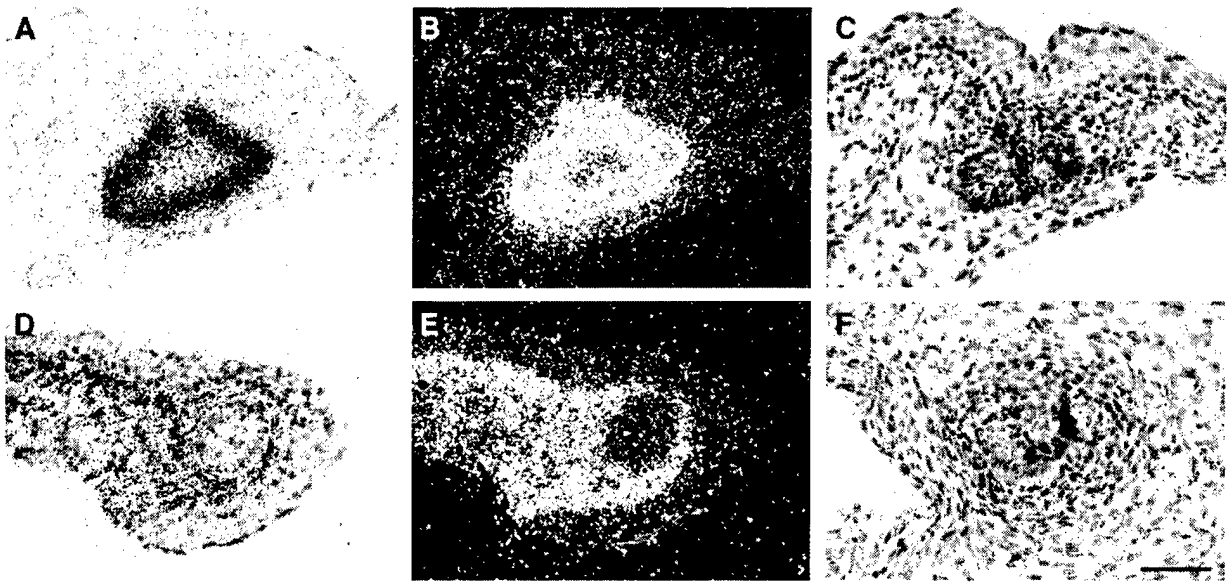

Fig. 4. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. (A)–(C). *In situ* hybridization for PTHrP mRNA in normal mammary rudiments at E16. (A) and (B) are brightfield and darkfield images, respectively, of the same section hybridized with antisense probe. (C) is a brightfield image of a similar section hybridized to sense probe as a control. Note that PTHrP mRNA is found in the mammary epithelial cells, especially those located peripherally. There is no hybridization within the mesenchyme. Note the lack of hybridization of the sense probe (compare (A) and (C). (D)–(F). *In situ* hybridization for PTH/PTHrP receptor mRNA in normal mammary rudiments at E15. (D) and (E) are brightfield and darkfield images, respectively, of the same section hybridized with antisense probe. Note that PTH/PTHrP receptor mRNA is found within the dense mammary and dermal mesenchyme; there is no receptor mRNA expressed within the mammary epithelial cells. (F) is a brightfield image of a similar section hybridized to PTH/PTHrP receptor sense probe as a control. Note the lack of signal as compared to (D). Scale bar represents 15 microns for all panels. [Reprinted with permission from (33).]

PTHrP expression is especially intense in the cells located peripherally, adjacent to the basement membrane. It is also expressed in developing hair follicles, but there appears to be minimal expression within the embryonic epidermis in general. In contrast to the epithelial pattern of PTHrP expression, PTH/PTHrP receptor expression is restricted to mesenchymal cells (Fig. 4D–4F). At E12, PTH/PTHrP receptor mRNA is expressed throughout the ventral dermis, including the dense mammary mesenchyme. At E18, as the mammary ducts grow and invade the developing fatty stroma, PTH/PTHrP receptor mRNA expression continues to be expressed in stromal cells, including those immediately surrounding the growing mammary duct. Therefore, within the embryonic mammary gland, it appears that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal signaling circuit in which PTHrP is produced by mammary epithelial cells and interacts with its receptor on mammary mesenchymal cells. In the context of the phenotypes of the PTHrP and PTH/PTHrP knockout mice, this would suggest that PTHrP is an epithelial signal that modulates stromal cell function in a fashion that is critical for its ability to support further morphogenesis of the mammary bud. Although the dense mammary mesenchyme appears structurally intact in PTHrP and PTH/PTHrP receptor knockout embryos, it is clearly functionally deficient. Based on these observations, our current working hypothesis is that PTHrP is necessary for the proper "functional differentiation" of the dense mammary mesenchyme.

The Role of PTHrP During Puberty and Early Pregnancy

Following birth, the murine mammary gland undergoes little development until the onset of puberty at 3–4 weeks of age (38). At that point, under hormonal influence, the distal ends of the mammary ducts form specialized structures called terminal end buds, which serve as the sites of active cellular proliferation and differentiation during ductal growth and morphogenesis (43). By 8–10 weeks of age, the epithelial duct system has grown to the borders of the mammary fat pad and the terminal end buds disappear, leaving the

typical branched duct system found in the adult virgin gland. After the onset of pregnancy there is another round of epithelial proliferation that leads to the production of terminal ducts, and then the formation of lobulo-alveolar structures. It appears that, in addition to embryonic development, PTHrP may also play a role in the epithelial/mesenchymal interactions governing these later rounds of ductal morphogenesis.

Within the mature mammary gland, PTHrP was first noted to be expressed during late pregnancy and lactation (44,45). More recently, our laboratory has shown that the PTHrP and PTH/PTHrP receptor genes are also expressed during puberty and early- to mid-pregnancy (42). Similar to our observations during embryonic development, at these time points we found PTHrP mRNA expressed in epithelial cells and PTH/PTHrP receptor mRNA expressed in stromal cells. However, compared to embryonic development, we found the overall levels of expression of both mRNA's to be less intense in the post-natal gland. In addition, during puberty, expression of both the PTHrP and PTH/PTHrP receptor genes appeared to be most prominent in and around terminal end buds. By *in situ* hybridization, we found no PTHrP mRNA in mature mammary ducts, only within epithelial cells located in end buds (Fig. 5A–5C). During early pregnancy, we could detect a low level of PTHrP mRNA in the epithelial cells of the developing lobuloalveolar units. We found a low level of PTH/PTHrP receptor mRNA within the stroma of the fat pad and within the periductal stroma of mature ducts during puberty and during early pregnancy, but, during puberty, there was also intense receptor mRNA expression within the stromal cells immediately surrounding terminal end buds (Fig. 5D–5F). These studies demonstrate that during the post-natal phases of active mammary ductular morphogenesis, the PTHrP gene is expressed in epithelial cells, and the PTH/PTHrP receptor gene is expressed in mammary stromal cells. Furthermore, expression of both genes is most intense in regions of the mammary gland that are actively proliferating and undergoing ductal morphogenesis, the terminal end buds.

Consistent with these observations, studies in transgenic mice have shown that overexpression of PTHrP in mammary myoepithelial cells, driven by the keratin 14 (K14) promoter, results in abnormal mammary ductal development during puberty and early pregnancy (Fig. 6) (46). During puberty, PTHrP-overexpression resulted in severe impairments in both the overall rate of ductal proliferation as well as in the pattern of side branching. This, in turn, caused a delay in ductal growth into the mammary fat pad and resulted

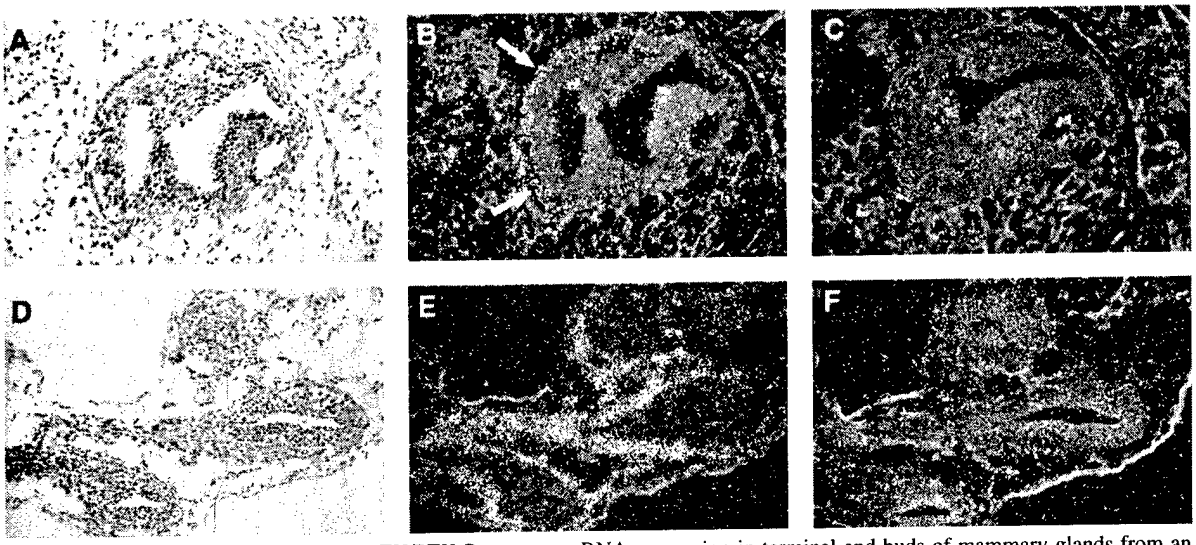

Fig. 5. Localization of PTHrP and the PTH/PTHrP receptor mRNA expression in terminal end buds of mammary glands from an adolescent (4-week-old virgin) mouse. (A)–(C) *In situ* hybridization for PTHrP mRNA in terminal end buds. A and B represent brightfield and darkfield images, respectively, of a section through a terminal end bud hybridized with an antisense probe. (C) represents a darkfield image of an adjacent section hybridized with a sense probe as a control. (D)–(F) *In situ* hybridization for PTH/PTHrP receptor mRNA in terminal end buds. (D) and (E) represent brightfield and darkfield images, respectively, of a section through terminal end buds hybridized with an antisense probe. (F) represents a darkfield image of an adjacent section hybridized with a sense probe as a control. Note that PTHrP mRNA expression is localized to epithelial cells of terminal end buds, whereas PTH/PTHrP receptor mRNA expression is localized to stromal cells immediately surrounding terminal end buds.

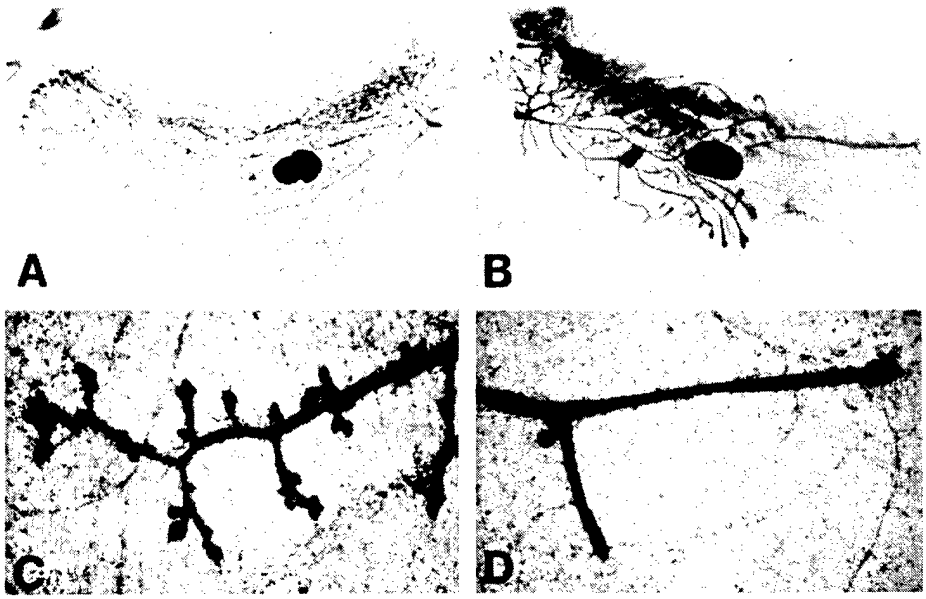

Fig. 6. Whole mount analysis of mammary glands from 6-week-old PTHrP-overexpressing mice. Mammary glands from 6-week-old transgenic mice (B) and (D) and a control littermate (A) and (C) were resected in toto and stained with carmine aluminum. A and B represent low-power views of whole mounts of the #4 inguinal mammary gland from normal (A) and transgenic (B) mice. (C) and (D) represent high-power views of the same samples. Note the overall retarded growth of the ducts into the fat pad of the transgenic gland (B). Also, note the simpler ductal architecture with many fewer branches in transgenic (D) as compared to normal (C) glands. [Reprinted with permission from (40).]

in a much simpler overall duct structure in transgenic mice. In addition, PTHrP-overexpression resulted in an impairment of terminal duct development during early pregnancy. These effects were mediated by amino-terminal PTHrP acting through the PTH/PTHrP receptor, because overexpression of PTH produced a similar phenotype and local administration of PTHrP(1–36) within the fat pads of normal mice also impaired ductular proliferation (40). In the context of the expression patterns described within the previous paragraph, these studies suggest that PTHrP continues to participate in epithelial-mesenchymal interactions in the post-natal mammary gland and may contribute to the regulation of ductal branching morphogenesis during puberty by regulating proliferation and/or morphogenesis of the terminal end buds.

PTHrP Regulates Mammary Stromal Cell Function During Ductal Morphogenesis

These experiments suggest that PTHrP, produced by mammary epithelial cells, acts to modulate stromal cell function at several different phases of ductal morphogenesis. Our current hypothesis is that PTHrP interacts with the PTH/PTHrP receptor on mammary stromal cells and that this interaction is critical to the ability of the stroma to support proper ductal branching morphogenesis. In addition to the phenotypes of PTHrP-knockout and overexpressing mice detailed earlier, several experiments support this hypothesis. First, we found that primary cultures of mouse mammary stromal cells expressed PTH/PTHrP receptor mRNA, but no PTHrP mRNA. Conversely, mammary epithelial cells from freshly isolated organoids, expressed PTHrP mRNA but not PTH/PTHrP receptor mRNA. These results lend further support to the epithelial/mesenchymal pattern of expression of PTHrP and PTH/PTHrP receptor in the mammary gland. In addition, mammary stromal cells, in culture, bound amino-terminal PTHrP and responded with an increase in intracellular cAMP (42). Together, these observations confirm that mammary stromal cells contain functional PTH/PTHrP receptors. Finally heterotypic tissue recombination experiments have documented that PTHrP signaling is necessary for mesenchymal cells to support epithelial cell survival and morphogenesis (42). Mammary epithelium from PTH/

PTHrP receptor knockout embryos was able to survive and initiate ductal morphogenesis when combined with normal mesenchyme and transplanted beneath the kidney capsule of athymic mice. However, mesenchymal cells from PTH/PTHrP receptor knockout embryos were not able to support the survival or outgrowth of either receptor knockout epithelium or normal epithelium. These results support the idea that stromal cells must interact with PTHrP in order to support normal epithelial morphogenesis.

The molecular mechanisms by which PTHrP exerts its effects on mammary stromal cells remain unclear. Studies have shown that the stroma secretes growth factors that regulate epithelial morphogenesis (47–50). In addition, the stroma contributes to the extracellular matrix, whose composition can have profound influences on epithelial cell behavior (38,51). The mammary stroma is also the source of matrix mettalloproteinases that are involved in remodeling the ECM during ductal branching morphogenesis (52–54). All of these molecules are potential downstream stromal effectors of PTHrPs actions on epithelial development, and experiments are currently underway to examine PTHrPs effects on their expression in cultured mammary stromal cells. However, whatever the exact nature of the stromal response to PTHrP, these experiments underscore the reciprocal nature of the epithelial mesenchymal interactions regulating epithelial morphogenesis. It is well established that stromal cells influence the form and function of the epithelium. However, the epithelial cells are equal partners in this task, for without epithelial signals, such as PTHrP, stromal cells cannot properly direct epithelial morphogenesis.

PTHrPs Role in Breast Cancer

It has long been appreciated that breast cancer cells have a propensity to metastasize to bone (55). This "osteotrophism" results in an incidence of skeletal metastases as high as 70% in series of women with advanced disease (56). A significant proportion of these patients also develop hypercalcemia, both due to local bone destruction or osteolysis as well as in association with classical HHM (1,56). It has been known that PTHrP was the cause of humoral hypercalcemia associated with breast cancer for some time, but recent data suggest that this molecule might also be involved in the local osteolysis associated with these tumors and, in fact, may play an important role in the osteotrophism itself.

PTHrP has now been reported to be produced by both cultured breast cancer cell lines as well as by primary human breast cancers (56). Several histological surveys have demonstrated that about 50–60% percent of primary breast tumors express PTHrP (57–59). In addition, a series of observations has demonstrated that PTHrP is more commonly expressed in breast cancer metastatic to bone as compared to other sites of metastasis or within the primary tumor itself, as determined by both immunohistochemistry and *in situ* hybridization (60,61). These observations suggest that either PTHrP expression within a given population of tumor cells might be involved in establishing the bony metastasis, or, alternatively, that once within the bone microenvironment, tumor cells upregulate PTHrP expression. There is evidence to support both of these possibilities. First, several clinical studies have shown that PTHrP expression within a primary breast tumor is predictive of bony metastases (56–59). Second, in an elegant series of animal experiments, Guise and colleagues have demonstrated that levels of PTHrP expression within breast cancer cells influenced the likelihood of these cells metastasizing to bone. When introduced into the left ventricle of nude mice, MDA-MB-23 cells engineered to overexpress PTHrP produced 3 times more bone metastases than native MDA-MB-23 cells, which express low levels of PTHrP (62). In addition, despite the low levels of PTHrP produced by native MDA-MB-23 cells, treatment of the mice with PTHrP antisera was able to reduce the number and size of bone metastases produced by these cells by up to five-fold (63). The combination of these clinical and animal data strongly suggest that PTHrP production facilitates the establishment of breast cancer cells within the skeleton.

If PTHrP is involved in the osteotrophism of breast cancer cells, it most likely does so through its ability to activate osteoclastic bone resorption. In order for a metastatic cell to take up residence in bone, it must be able to recruit resident osteoclasts to resorb mineralized tissue (56). This osteolysis releases a series of growth factors from the bone matrix that, in turn, nourish the tumor cells, giving them a growth advantage and causing more local bone resorption (56). It is thought that this vicous cycle underlies the pernicious behavior of breast cancer metastatic to bone and, as a proof of concept, several large clinical trials have now demonstrated that pharmacologic inhibition of osteoclastic bone resorption inhibits both the occurrence

and growth of bony metastases (64, 65). There is evidence from the Guise group that upregulation of PTHrP expression in breast cancer cells by TGF-β within the bone environment may play a role in the establishment of the vicous cycle described above. TGF-β has been shown to upregulate PTHrP expression in breast cancer cell lines (4); it is plentiful within the bone matrix and it is released into the skeletal microenvironment upon osteoclastic bone resorption (66). By introducing a mutant, dominant negative TGF-β type II receptor into MDA-MB-23 cells, Guise and colleagues were able to abolish the upregulation of PTHrP by TGF-β and to reduce the occurrence and growth of bone metastases in their animal model (67).

Breast cancer cells appear to activate bone resorption both by increasing the numbers of osteoclasts in their immediate vicinity and by increasing the resorbing activity of individual cells (68). Preliminary evidence from the laboratory of Dr. Karl Insogna suggests that PTHrP production may be involved in the ability of breast cancer cells to support osteoclast recruitment. This group has demonstrated that breast cancer cells can support the differentiation of osteoclasts from bone marrow stroma in cell culture and it appears that PTHrP is the cytokine that correlates most closely with the ability of individual cell lines to support osteoclast differentiation (Sun and Insogna, unpublished data). It is interesting to note here that the differentiation of osteoclasts from their monocytic precursors is normally dependent on an interaction between these cells and cells of the bone marrow stromal lineage. Furthermore, it has been suggested that PTHrP and PTH promote osteoclast differentiation indirectly by acting on these stromal-derived cells (69). Hence, there may well be fundamental similarities in the mechanisms underlying PTHrPs regulation of mammary stromal cell function during development and its actions to promote bony metastasis in breast cancer.

In summary, the hypothesis emerging from these data is that PTHrP, by virtue of its ability to activate osteoclastic bone resorption, helps a breast cancer cell obtain a foothold in bone. Once established in the skeleton, the tumor cells, responding to factors like TGF-β that are released from the bone matrix, upregulate their production of PTHrP, which contributes to further bone resorption and the continued growth of the tumor cells, clearly a vicious cycle. The mechanisms by which PTHrP upregulates bone resorption remain an area of active research, but one possibility may be the ability of PTHrP to promote osteoclast differentiation. Whatever the exact mechanisms by which PTHrP acts, if it is true that this cytokine is critical to the osteotrophism of breast cancer cells, then PTHrP and its receptor(s) may be useful therapeutic targets for therapy aimed at preventing or treating bone metastases from breast tumors.

CONCLUSIONS

PTHrP appears to play a role in epithelial-stromal interactions in normal mammary gland development and in breast cancer. During development, it is a necessary participant in the regulation of mammary mesenchymal cell function during embryogenesis, and it appears to continue to participate in epithelial-mesenchymal interactions that regulate the later stages of ductal morphogenesis as well. In breast cancer, it appears to influence the interactions of malignant cells with the skeletal microenvironment and bone marrow stroma in such a way as to contribute to the osteotrophism that makes metastatic breast cancer such a devastating disease. It is likely that further research into the effects of PTHrP on mammary stromal cells will help to shed light on epithelial-mesenchymal interactions important to both mammary development and neoplasia. It will be particularly interesting to determine if PTHrP contributes to the regulation of tumor growth or early events in the metastatic cascade by influencing tumor-stromal interactions at the site of the primary tumor. It is our hope that ongoing studies will provide answers to these questions as well as continuing to broaden our understanding of both PTHrP and mammary development.

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Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development

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SUMMARY

Parathyroid hormone-related protein (PTHrP) was originally discovered as a tumor product that causes humoral hypercalcemia of malignancy. PTHrP is now known to be widely expressed in normal tissues and growing evidence suggests that it is an important developmental regulatory molecule. We had previously reported that overexpression of PTHrP in the mammary glands of transgenic mice impaired branching morphogenesis during sexual maturity and early pregnancy. We now demonstrate that PTHrP plays a critical role in the epithelial-mesenchymal communications that guide the initial round of branching morphogenesis that occurs during the embryonic development of the mammary gland. We have rescued the PTHrP-knockout mice from neonatal death by transgenic expression of PTHrP targeted to chondrocytes. These rescued mice are devoid of mammary epithelial ducts. We show that disruption of the PTHrP gene leads to a failure of the initial round of branching growth that is responsible for transforming the mammary bud into the rudimentary mammary duct system. In the absence of PTHrP, the mammary epithelial cells degenerate and disappear. The ability of PTHrP to support embryonic mammary development is a function of amino-terminal PTHrP, acting via the PTH/PTHrP receptor, for ablation of the PTH/PTHrP receptor gene recapitulates the phenotype of PTHrP gene ablation. We have localized PTHrP expression to the embryonic mammary epithelial cells and PTH/PTHrP receptor expression to the mammary mesenchyme using in situ hybridization histochemistry. Finally, we have rescued mammary gland development in PTHrP-null animals by transgenic expression of PTHrP in embryonic mammary epithelial cells. We conclude that PTHrP is a critical epithelial signal received by the mammary mesenchyme and involved in supporting the initiation of branching morphogenesis.

Key words: Epithelial-mesenchymal interaction, Branching morphogenesis, Ectodermal dysplasia, Genetic rescue, Keratin 14, Organogenesis, PTH/PTHrP receptor, Mammary mesenchyme

INTRODUCTION

Parathyroid hormone-related peptide (PTHrP) was initially isolated from tumors causing the paraneoplastic syndrome of humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). Its name reflects the fact that PTHrP and parathyroid hormone (PTH) are the products of genes that have diverged from a common ancestor (Broadus and Stewart, 1994). Unlike PTH, which is produced only by the parathyroid glands and circulates as a classic peptide hormone that regulates systemic calcium metabolism, PTHrP is produced by a wide variety of fetal and adult tissues, does not circulate and exerts its actions locally (Broadus and Stewart, 1994). PTH and PTHrP retain a high degree of homology in their amino-terminal portions, and PTH and amino-terminal species of PTHrP have retained the use of a common G-protein-coupled receptor, the PTH/PTHrP receptor (Jüppner et al., 1991). PTHrP has also been shown to undergo post-translational processing to generate several other peptides, at least one of which has been demonstrated to have biological activity subserved by an as yet unidentified receptor distinct from the PTH/PTHrP receptor (Wu et al., 1996; Kovacs et al., 1996).

PTHrP has been implicated in the regulation of a variety of biological processes such as cell growth and differentiation, the regulation of pancreatic islet cell function, the regulation of smooth muscle tone and the facilitation of placental calcium transport (Philbrick et al., 1996). Although the exact physiological functions of PTHrP remain unclear in mature organisms, a series of recent experiments in transgenic mice has demonstrated that PTHrP serves important roles during fetal development. Disruption of the PTHrP and PTH/PTHrP receptor genes and overexpression of PTHrP in chondrocytes have shown that PTHrP regulates chondrocyte differentiation during endochondral bone formation (Karaplis et al., 1994; Vortkamp et al., 1996; Lanske et al., 1996; Weir et al. 1996). In the absence of PTHrP or the PTH/PTHrP receptor, chondrocytes appear to differentiate and ossify prematurely, resulting in a chondrodystrophy that leads to the neonatal death of the knockout mice. Overexpression of PTHrP in chondrocytes leads to the opposite phenotype, a profound delay in the differentiation of chondrocytes resulting in the birth of mice with a cartilaginous skeleton. In addition to effects in chondrocytes, PTHrP has been implicated as playing a role in epithelial-mesenchymal interactions during hair follicle and mammary gland development. Overexpression of PTHrP in keratinocytes (Wysolmerski et al., 1994) results in either a delay or failure of hair follicle initiation, and its overexpression in mammary myoepithelial cells (Wysolmerski et al., 1995) has been shown to impair mammary ductal development.

Shortly after its discovery, PTHrP mRNA was found to be expressed in the lactating mammary gland and PTHrP was found in high concentrations in milk (Thiede and Rodan, 1988; Budayr et al., 1989). The role of PTHrP during lactation remains obscure, but it is now clear that PTHrP is expressed at various stages during mammary gland development, and overexpression of PTHrP in myoepithelial cells has been shown to retard ductular growth and to impair side branching during sexual maturation as well as to inhibit the formation of terminal ductules during early pregnancy (Wysolmerski et al., 1995). In addition, PTHrP introduced directly into the mammary fat pads of normal mice has been shown to impair estrogen- and progesterone-induced ductular proliferation (Wysolmerski et al., 1995). Because the skeletal phenotypes of PTHrP underexpression and overexpression were exact opposites of each other, we hypothesized that PTHrP gene ablation might also lead to defects in ductular growth and or branching. However, because mammary development occurs to a great extent after birth, and because the PTHrP-knockout mice die at birth, in order to test this hypothesis, we needed to devise a strategy to rescue these mice from their neonatal demise. In this report, we describe our strategy for rescuing the PTHrP-null mice, and we demonstrate that PTHrP is essential for mammary gland development.

MATERIALS AND METHODS

Mouse strains and identification of knockout embryos.

The disrupted PTHrP allele (Karaplis et al., 1994) was progressively outbred onto a CD-1 background and mice heterozygous for this allele were mated to produce PTHrP-null embryos. The date of the appearance of a vaginal plug was considered to be day 0 of embryonic life. Embryos were removed from the uterus and genotyped with respect to the presence or absence of neomycin gene sequences and the presence or absence of an intact PTHrP-coding region (exon IV) by PCR, utilizing the following primer sets: wild-type murine PTHrP gene – forward 5′-GCTACTGCATGACAAGGGCAAGTCC and reverse 5′-CATCACCCACAGGCTAGCGCCAACT (421 bp product), and bacterial neomycin gene – forward 5′-GGAGAGGCTATTCG-GCTATGAC and reverse 5′-CGCATTGCATCAGCCATGATGG (315 bp product). This allowed the identification of wild-type, heterozygous and homozygous PTHrP-null embryos. The disrupted PTH/PTHrP receptor allele was progressively bred onto a Black Swiss background, and homozygous-null embryos were produced and identified in like fashion (Lanske et al., 1996). The PTH/PTHrP receptor primer pair utilized for this purpose amplified a 270 bp portion of the PTH/PTHrP receptor gene and consisted of the following sequences: forward 5′GCAGAGATTAGGAAGTCTTGGA and reverse 5′AGCCGTCGT-CCTTGGGAACTGT.

Col II-PTHrP/PTHrP-null mice were produced in the following fashion. The col II-PTHrP and PTHrP-null alleles were first bred onto a CD-1 background for several generations to minimize any potential exacerbating effects of their original different genetic backgrounds. Then col II-PTHrP transgenic mice were crossed to PTHrP-null heterozygotes to generate offspring carrying both the transgene and a PTHrP-null allele. These were again crossed to PTHrP-null heterozygotes to generate col II-PTHrP hemizygous, PTHrP-null homozygous mice (col II-PTHrP/PTHrP-null mice). The PTHrP-null allele was identified as outlined above. The col II-PTHrP transgene was identified in like fashion using the following primers that identified a 510 bp section of the murine procollagen II promoter/human PTHrP cDNA junction segment: forward 5′-TCTT-AGCATTCTTGGAGAAC and reverse 5′-ATCAGATGGTGAAGG-AAG.

K14-PTHrP/PTHrP-null embryos were produced by mating K14-PTHrP transgenic hemizygotes (Wysolmerski et al., 1994) with mice heterozygous for the PTHrP-null mutation. Offspring of this cross that were both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null gene were then crossed to mice heterozygous for the PTHrP-null allele to produce mice homozygous for a disrupted PTHrP gene and hemizygous for the K14-PTHrP transgene. The K14 transgene was identified as previously described (Wysolmerski et al., 1995).

K14-PTHrP, col II-PTHrP/PTHrP-null (double rescue) mice were produced as follows. We first created K14-PTHrP hemizygous, PTHrP-null heterozygous and col II-PTHrP hemizygous, PTHrP-null heterozygous mice as described above. These mice were then crossed to one another to generate K14-PTHrP/col II-PTHrP double transgenic, PTHrP-null homozygotes (double rescue) mice. The various alleles were identified as outlined above.

Each of the various types of embryos was also sexed based on the presence or absence of a 240 bp band amplified from the SRY gene using the following primers: forward 5′-CGG-GATCCATGTCAAGCGCCCCATGAATGCATTTATG and reverse 5′-GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT (Geise et al., 1994).

Histology/immunohistochemistry

Embryos were harvested by caesarean section and fixed in 4% paraformaldehyde at 4°C for 12 hours. The ventral skin was then removed, and the embryonic mammary glands were identified using transmitted light and photographed under low magnification. Subsequently, the mammary glands were dissected from the ventral skin and embedded in paraffin. Serial 5 μm sections were cut and stained with hematoxylin and eosin for microscopic examination. Immunohistochemistry was performed using standard techniques. The mouse casein antibody is a rabbit polyclonal antibody (kind gift of B. Vonderhaar, NIH, Bethesda MD) and was used at a dilution of 1:200. All primary incubations were performed for 12 hours at 4°C,

and primary antibody binding was detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and 3, 3′ diaminobenzidine as a chromagen. Slides were counterstained using hematoxylin. Apoptosis was detected by terminal deoxytransferase labelling (TUNEL assay) employing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Mannheim, Germany).

In situ hybridization histochemistry

In situ hybridization histochemistry was performed on 5 μm paraffin sections of embryonic mammary glands as follows. Probes corresponded to a 349 bp genomic fragment of the mouse PTHrP gene and a 238 bp cDNA fragment of the PTH/PTHrP receptor gene, as previously described (Weir et al., 1996). Sense and antisense riboprobes were generated from linearized fragments using an in vitro transcription kit (Promega, Madison, WI) in the presence of ^{35}S-UTP (1000 Ci/mmol, Amersham, Life Science, Arlington Heights, IL). Before hybridization, sections were dewaxed and rehydrated, treated with proteinase K (3 μg/ml in PBS for 17 minutes at room temperature), and acetylated with 0.25% acetic anhydride in the presence of 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 minutes. Sections were then rinsed in 2× SSC and incubated for 30 minutes in 0.66% N-ethylmaleimide (Sigma Chemical Co., St Louis, Mo) in 2× SSC, rinsed again in 2× SSC, dehydrated in graded alcohol, treated with chloroform for 5 minutes, rehydrated and then air dried. The probes (1.5×10^7 cts/minute/ml) were then hybridized to the samples for 17 hours at 54°C in a humidified chamber. Hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 250 μg/ml tRNA, 100 μg/ml salmon sperm DNA and 50 mM DTT. After hybridization, sections were rinsed in 1× SSC and washed twice in 2× SSC/50% formamide for 5 minutes at 52°C, rinsed in 2× SSC, and treated with 30 μg/ml RNase A in 2× SSC at 37°C for 30 minutes. Following two rinses in 2× SSC, sections were again washed in 2× SSC/50% formamide at 52°C for 5 minutes, dehydrated through graded ethanol, air dried and dipped in a 1:1 mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development, sections were counterstained with hematoxylin and mounted for microscopic examination.

RESULTS

Col II-PTHrP rescued PTHrP-null mice lack mammary glands

Disruption of the PTHrP gene by homologous recombination resulted in defects in skeletal development including inappropriate ossification of the costal cartilage, resulting in a shield chest and respiratory failure (Karaplis et al., 1994). Most other tissues appear to have developed normally in these mice, but the neonatal death of these animals had precluded a full examination of the role of PTHrP in sites, such as the mammary gland, which develop after birth. Because overexpression of PTHrP via a procollagen II-PTHrP (col II-PTHrP) transgene produced a skeletal phenotype reciprocal to that seen in the PTHrP-knockout mice (Weir et al., 1996; Karaplis et al., 1994), we reasoned that delivery of PTHrP to chondrocytes, via this transgene, might rescue the skeletal phenotype of the PTHrP-knockout mice and allow these animals to survive beyond birth. Our goal was to produce a mouse that lacked PTHrP in all tissues except cartilage, where it would be supplied by the col II-PTHrP transgene. To this end, we bred the col II-PTHrP transgene onto a PTHrP-null background to produce col II-PTHrP/PTHrP-null mice. These mice survived to maturity but suffered from multiple abnormalities including defects in the integument and its appendages, and failures of tooth eruption and mammary epithelial development, a phenotype reminiscent of the collection of human syndromes known as ectodermal dysplasias (Freire-Maia and Pinheiro, 1994). In this report, we detail the effects of the loss of PTHrP on mammary development.

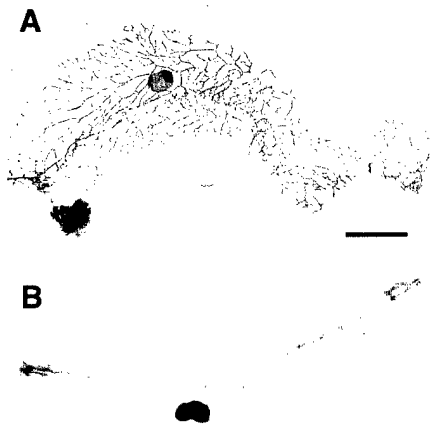

Fig. 1. Whole-mount analysis of mammary glands from col II-PTHrP/PTHrP-null mice and normal littermates. The fourth inguinal mammary glands were resected from 4-month-old normal and col II-PTHrP/PTHrP-null mice, fixed in acid ethanol and stained with carmine aluminum. The normal gland (A) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In contrast, the col II-PTHrP/PTHrP-null gland (B) is devoid of epithelial structures; only the lymph node and vasculature are present within the fat pad. These results are representative of the findings in 2 col II-PTHrP/PTHrP-null females. Scale bar represents 5 mm.

Fig. 1 demonstrates the morphology of whole mammary glands taken from 4-month-old, female col II-PTHrP/PTHrP-null (Fig. 1B) and normal littermate (Fig. 1A) mice. As one can see, the mature virgin mammary gland (Fig. 1A) consists of a series of branched epithelial ducts filling out a specialized stromal compartment known as the mammary fat pad. In contrast, col II-PTHrP/PTHrP-null mice lacked any evidence of mammary epithelial ducts (Fig. 1B). The mammary fat pad and its vasculature appeared to form normally, but were devoid of any mammary epithelium. Furthermore, examination of the ventral epidermis failed to demonstrate any nipple structures. These data suggested that PTHrP is essential for the development of the mammary epithelial duct system and nipples.

Loss of PTHrP results in a failure of the mammary epithelial primary growth spurt

The formation of the embryonic murine mammary gland is essentially a two-step process. The first step, occurring between E10 and E12, is the formation of the mammary buds. In female mice, the mammary buds remain relatively quiescent until E16 when they begin the second step, an initial round of branching morphogenesis, which leads to the formation of a mammary duct system with approximately 15-20 branches by

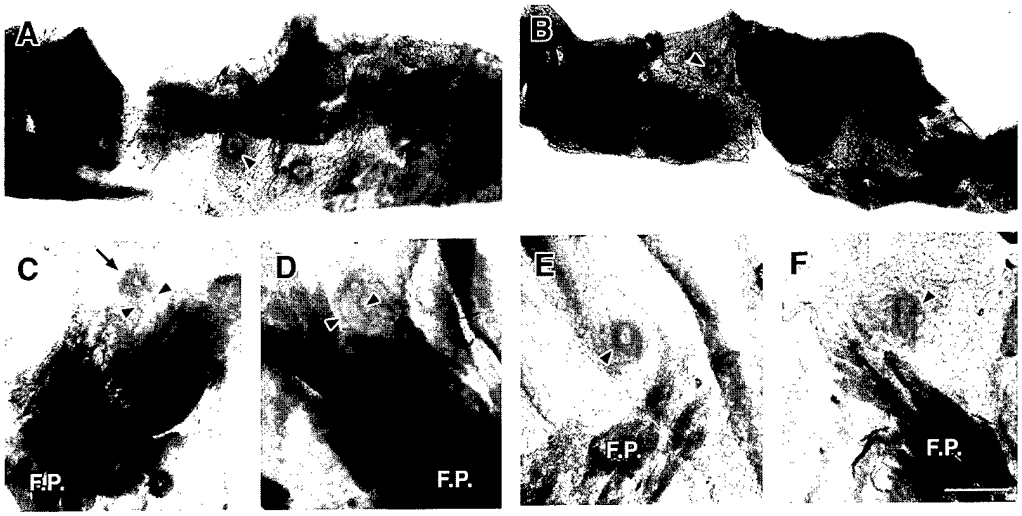

Fig. 2. Comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. The ventral epidermis was dissected from the respective embryos and photographed under low magnification using transmitted light in order to examine the gross structure of the embryonic mammary glands. (A,B) The mammary buds appear as round structures projecting upwards from the undersurface of the epidermis (arrowheads). Note that at E15, the mammary buds in the knockout embryos (B) appear similar to those in the normal embryos (A) (7 PTHrP-null female embryos analyzed, 6 wild-type embryos analyzed). In contrast, by E18 there is a dramatic difference in the appearance of the mammary structures in knockout (E,F) as compared to normal (C,D) embryos. At this point, the normal mammary structure consists of a developing nipple (dark halo, arrow in C) and an elongated primary duct (translucent tube-like structure between arrowheads in C,D) which is just beginning to form initial branches as it makes contact with the developing mammary fat pad (labelled F.P.). Note that in the PTHrP-knockout embryos (E,F), the mammary glands fail to elongate and remain bud-like (E) or slightly ectatic (F) in their appearance. There are no primary ducts that extend to the fat pads, and the developing fat pads (F.P.) themselves appear diminished in size (13 PTHrP-null female embryos examined, 6 wild-type embryos examined). Scale bar represents 160 μm for all panels.

birth (Sakakura, 1987). The nipples of mice are formed on or about E18 as a circular invagination of the epidermis, referred to as the nipple sheath (Sakakura, 1987). Because col II-PTHrP/PTHrP-null mice lack nipples and given the timing of nipple formation at E18, we reasoned that the loss of epithelial ducts resulting from the lack of PTHrP most likely occurred during the embryonic development of the mammary gland. Therefore, we returned to the original PTHrP-knockout embryos and examined embryonic mammary gland growth at days E12-13, E15 and E18, and at birth.

Figs 2 and 3 demonstrate the gross and microscopic appearance, respectively, of the mammary rudiments from mice homozygous for a disrupted PTHrP gene as compared to their wild-type littermates. As shown in Figs 2A,B and 3A,B, the mammary buds appeared normal in PTHrP-null embryos at E15. This was also the case at E12-13 (data not shown). In contrast, there was a dramatic difference in the appearance of the PTHrP-knockout ducts as compared to those in wild-type embryos at E18. As seen in Fig. 2C,D, at this age in the normal embryos, the mammary bud has given rise to a primary duct, which has elongated to make contact with the developing mammary fat pad and has formed several initial branches. In the knockout embryos, the mammary buds failed to make this transition and appeared similar to those at E15 (Fig. 2E,F). Furthermore, the mammary fat pads, although present, appeared somewhat diminished in size in the knockout embryos.

On microscopic examination, one could see that, by E18, the normal ducts had extended into the lower dermis and had formed initial branches that could be seen amongst the preadipocytes constituting the developing mammary fat pad (see Fig. 3C,D). At this point, the normal mammary glands also had well-developed nipple sheaths surrounding the origins of the primary ducts (Fig. 3C). In contrast, as seen in Fig. 3E, at E18 the PTHrP-knockout ducts appeared not to have undergone the primary growth spurt. Instead of extending to the fat pad and branching, epithelial ducts were uniformly found only in the upper portions of the dermis, where, typically, they were enveloped by a dense condensation of fibroconnective tissue. In addition, there was no evidence of nipple sheath development surrounding the origins of the epithelial ducts in the PTHrP-knockout embryos. When examined at higher magnification, the epithelial cells within the knockout ducts often appeared to be degenerating. As compared to normal epithelial cells (Fig. 3F), there was separation of the PTHrP-knockout epithelial cells (Fig. 3G) from the basement membrane, the cells borders were indistinct, and many nuclei appeared pyknotic. Consistent with this observation, by birth, there were only scattered remnants of degenerating mammary ducts that could be found on serial sectioning of the PTHrP-null embryos while, in wild-type embryos, the mammary ducts were firmly established within the mammary fat pad and had developed the expected branching pattern (data not shown). In summary, in the absence of PTHrP, mammary development proceeds normally through the mammary bud stage but subsequently falters as the buds fail to undergo the initial phase of branching morphogenesis and the mammary epithelial cells then degenerate.

PTHrP overexpression has been shown to delay chondrocyte differentiation and apoptosis, whereas disruption of the PTHrP

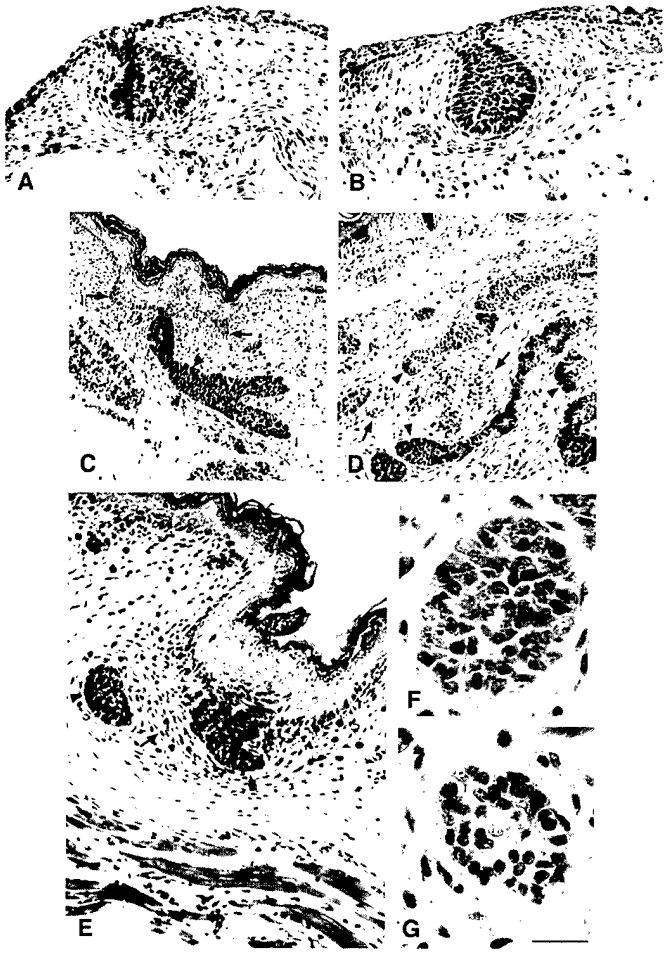

Fig. 3. Histologic comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. (A,B) Photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15 (3 knockout and 3 wild-type embryos examined). At this stage, the mammary bud consists of an invagination of mammary epithelial cells surrounded by a condensation of mammary mesenchyme, and the microscopic appearance of the PTHrP-knockout buds was entirely normal. (C-G) Photomicrographs of H&E-stained sections through mammary glands dissected from PTHrP-knockout (E,G) and normal littermate (C,D,F) embryos at E18 (5 knockout and 4 wild-type embryos examined). In a normal embryo (C,D) one can see the primary epithelial duct (arrowhead in C) arising from the epidermis and extending below the dermis where it branches (arrowheads in D) and makes contact with the preadipocytes (arrows in D) within the developing fat pad). In contrast, in the PTHrP-knockout embryos, (E) the epithelial duct (arrowheads) does not extend out of the upper regions of the dermis and becomes surrounded by an abnormally dense condensation of fibroconnective tissue (arrow in E). (F,G) High power photomicrographs of mammary epithelial ducts in cross-section taken from a normal (F) and PTHrP-knockout (G) embryo at E18. Note that, in the knockout duct (G), the epithelial cells appear to be degenerating; many nuclei are pyknotic, the cell cytoplasm appears reduced and somewhat vacuolated and the cells are separating from the basement membrane. Scale bar represents 16 μm in A,B; 25 μm in C,D; 17 μm in E; 5 μm in F,G.

gene results in a form of growth failure associated with premature differentiation and apoptosis of chondrocytes in the growth plate of developing bones (Weir et al., 1996; Amling et al., 1997; Karaplis et al., 1994; Lee et al., 1996; Vortkamp et al., 1996). Given these findings and the apparent degeneration of the mammary epithelial cells in the PTHrP-knockout embryos, we examined these cells for evidence of apoptosis and/or inappropriate differentiation at E18 by TUNEL assay and by immunohistochemistry for β-casein. Although apoptotic cells were easily detected in normal ducts, there were no apoptotic cells within the knockout ducts (data not shown). Likewise, immunohistochemistry for β-casein revealed no evidence that the knockout mammary epithelial cells were undergoing premature cytodifferentiation; there was no staining for β-casein in either normal or knockout epithelial cells at E18 (data not shown). These data suggest that, unlike the events in cartilage, the failure of mammary development in PTHrP-knockout mice appeared neither to be associated with premature differentiation of the mammary epithelial cells nor with widespread apoptosis of these cells.

Ablation of the PTH/PTHrP receptor gene recapitulates the mammary phenotype of PTHrP-knockout mice

As mentioned in the Introduction, PTHrP gives rise to several biologically active peptides (Broadus and Stewart, 1994; Wu et al., 1996). In addition, it has been suggested that PTHrP may be targeted to the nucleus and exert biological activity via an intracrine pathway (Henderson et al., 1995). Previous experiments had implicated soluble, amino-terminal PTHrP acting via the PTH/PTHrP receptor as important in the regulation of branching morphogenesis in the mammary gland during sexual maturation and pregnancy (Wysolmerski et al., 1995). In order to determine if this was also the case during embryogenesis, we examined mammary gland development in PTH/PTHrP receptor-null embryos (Lanske et al., 1996) over the same time frame as in the PTHrP-null embryos.

Fig. 4 demonstrates the appearance of the mammary rudiment in PTH/PTHrP receptor knockout mice and control littermates. As seen in the PTHrP-knockout embryos, in the receptor-knockout mice, the primary round of branching morphogenesis failed, leading to the subsequent degeneration of the mammary epithelial ducts. Just as with the PTHrP knockouts, the mammary buds appeared to form appropriately in the receptor knockout mice (data not shown), but clear differences in the appearance of the receptor-knockout mammary rudiment as compared to normal littermates were apparent by E18. As shown in Fig. 4, by E18, the normal duct system (Fig. 4A,D) had grown to the fat pad and begun to branch, while the knockout mammary duct failed to elongate or branch and remained bud-like in its appearance (Fig. 4B). Examination at higher magnifications revealed that the mammary ducts in the receptor knockout mice (Fig. 4C,E) remained in the upper dermis, were enveloped within an abnormal condensation of stroma and appeared to be degenerating, a picture nearly identical to that seen with mammary ducts devoid of PTHrP (see Fig. 3). Furthermore, as with the absence of PTHrP, the receptor knockout embryos formed no nipple sheath (see Fig. 4B). Therefore, ablation of PTHrP or the PTH/PTHrP receptor led to the same phenotype,

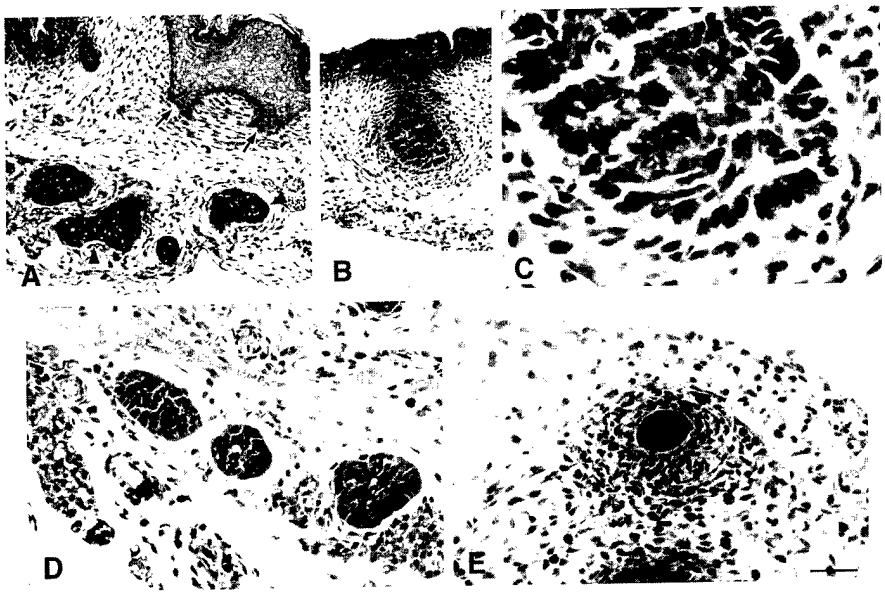

Fig. 4. Histologic comparison of the embryonic mammary glands of PTH/PTHrP receptor-knockout and normal littermate embryos at E18. (A) Photomicrograph of H&E-stained sections of mammary rudiment from a normal littermate. Note the initial branches of the primary duct (arrowheads) within the lower dermis. Also, note the developing nipple sheath (arrow) (3 embryos examined). (B) Photomicrograph of H&E-stained sections of mammary rudiment from PTH/PTHrP receptor-knockout embryo (5 receptor-knockout embryos examined). Note that the mammary duct has not elongated, that the mammary rudiment remains bud-like in its appearance and that there is no nipple sheath. (C) Higher magnification of B. Note that the epithelial cells appear to be degenerating; there are many pyknotic nuclei and the cell borders are indistinct, similar to the appearance of the PTHrP-knockout epithelial cells at this time point. (D,E) H&E-stained cross-sections of epithelial ducts from normal (D) and PTH/PTHrP receptor-knockout (E) mammary glands at E18. Note the lacy, delicate appearance of the stroma surrounding the normal ducts (D) as they make contact with the mammary fat pad. In contrast, note the condensation of stroma surrounding a rare PTH/PTHrP receptor-knockout duct (E) that has attempted to grow out from the mammary bud. Scale bar represents 20 μm in A,B; 4.5 μm in C; 10.4 μm in D,E.

a failure of the initial phase of branching morphogenesis during embryonic mammary development.

Localization of PTHrP and PTH/PTHrP receptor gene expression during embryonic mammary gland development

We next determined the sites of PTHrP and PTH/PTHrP gene expression in normal Balb/c mammary rudiments from E12 through E18 by in situ hybridization. As shown in Fig. 5A-C, PTHrP mRNA expression in the developing mammary rudiment was limited to the epithelial cells, especially those cells located peripherally, adjacent to the basement membrane. PTHrP mRNA was also detected in keratinocytes within the epidermis as well as within developing hair follicles, although it appeared that the highest levels of expression were within the mammary epithelial structures. Expression of the PTHrP

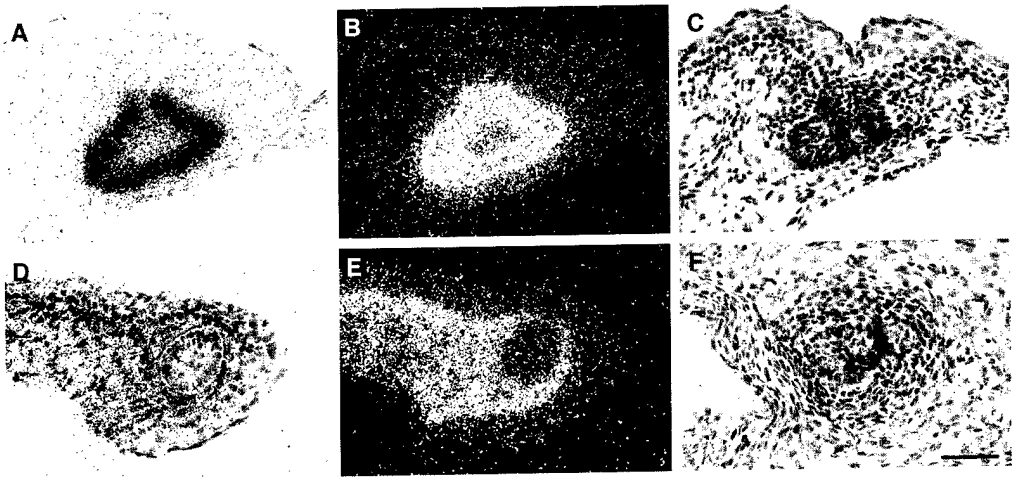

Fig. 5. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. (A-C) In situ hybridization for PTHrP mRNA in normal mammary rudiments at E16. (A,B) Bright-field and dark-field images, respectively, of the same section hybridized with antisense probe. (C) Bright-field image of a similar section hybridized to sense probe as a control. Note that PTHrP mRNA is found in the mammary epithelial cells, especially those located peripherally. There is no hybridization within the mesenchyme. Note the lack of hybridization of the sense probe (compare A and C). (D-F) In situ hybridization for PTH/PTHrP receptor mRNA in normal mammary rudiments at E15. (D,E) Bright-field and dark-field images, respectively, of the same section hybridized with antisense probe. Note that PTH/PTHrP receptor mRNA is found within the dense mammary and dermal mesenchyme; there is no receptor mRNA expressed within the mammary epithelial cells. (F) Bright-field image of a similar section hybridized to PTH/PTHrP receptor sense probe as a control. Note the lack of signal as compared to D. Scale bar represents 15 μm for all panels.

gene did not appear to be induced at any specific point during the time period that we examined (E12-18). Rather, PTHrP mRNA was continuously expressed at high levels in mammary epithelial cells in the mammary bud as well as in the growing ducts during the initial phase of branching morphogenesis.

In contrast to the epithelial expression pattern seen for PTHrP, expression of the PTH/PTHrP receptor was limited to the mesenchyme. As seen in Fig. 5D-F, PTH/PTHrP receptor mRNA was expressed throughout the embryonic dermis, including the dense mammary mesenchyme. At E12-13, the expression of the receptor mRNA appeared to be fairly uniform throughout the dermal mesenchyme (data not shown), but, from E15 onward, there appeared to be more intense hybridization of the receptor antisense probe in the upper, more cellular dermis (Fig. 5E). At E18, at a point at which the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in the stromal cells surrounding the growing mammary ducts as they became surrounded by the developing fatty stroma (data not shown). As with PTHrP gene expression, the PTH/PTHrP receptor gene was expressed throughout the time frame examined, and there was not a specific point at which its expression appeared to be induced. Therefore, within the embryonic mammary gland, PTHrP and the PTH/PTHrP receptor appear to represent an epithelial/mesenchymal signalling unit in which PTHrP is produced by mammary epithelial cells and interacts with its receptor on mammary mesenchymal cells.

Transgenic expression of PTHrP rescues the mammary glands of PTHrP-knockout mice

We hypothesized that the failure of mammary development seen in the PTHrP and PTH/PTHrP receptor-knockout embryos was due to the loss of PTHrP-mediated paracrine signalling between the mammary epithelium and mammary mesenchyme. This working hypothesis suggested that reintroducing PTHrP into the local microenvironment of the mammary bud might prevent the failure of mammary development in these mice. Keratin-14 expression is known to be induced in embryonic skin beginning at E15-16 (Kopan and Fuchs, 1989), about the time of the primary growth spurt of the mammary rudiment. Furthermore, the keratin-14 gene had been shown to be expressed in epithelial cells in the adult mammary gland (Smith et al., 1990; Wysolmerski et al., 1995). Therefore, we examined K14 expression in the embryonic mammary gland and found that it was expressed uniformly in embryonic mammary epithelial cells beginning on or about E15 (data not shown). Since we had shown that the K14-PTHrP transgene faithfully reproduced the native pattern of K14 expression in the mature mammary gland (Wysolmerski et al., 1995), we used this transgene as a vehicle to reintroduce PTHrP into the mammary environment of the PTHrP-null mice. We took a two-tiered approach. First, in order to ascertain if replacement of PTHrP into mammary epithelial cells rescued embryonic mammary development, we bred the K14-PTHrP transgene onto a homozygous PTHrP-null background to produce K14-PTHrP/PTHrP-null mice. These mice were devoid of PTHrP in all tissues except for those expressing the K14 gene. Second, in order to examine the effects of PTHrP replacement on the subsequent development of the mammary duct system within adolescent mice, we bred both the col II-PTHrP and K14-PTHrP transgenes onto a homozygous PTHrP-null background to produce col II-PTHrP, K14-PTHrP/PTHrP-null (double rescue) mice. These double-rescue mice lacked PTHrP in all tissues except for chondrocytes and sites of K14 expression.

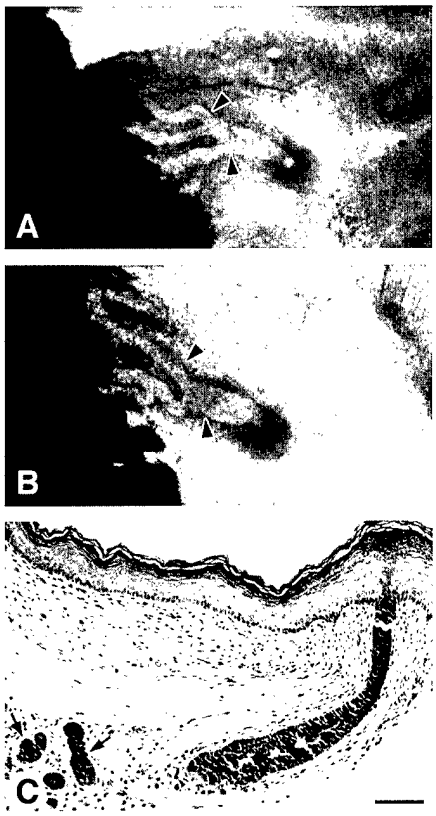

Fig. 6. Mammary development in K14-PTHrP/PTHrP-null mice. (A,B) Photographs of the whole mammary glands of a normal (A) and K14-PTHrP/PTHrP-null neonate (representative of 2 K14-PTHrP/PTHrP-null animals analyzed). The ventral skin was dissected and the mammary glands were viewed under low magnification using transmitted light. In both cases, the mammary gland consists of a primary duct (between arrowheads) that forms several branches before entering the mammary fat pad (dark area to the left). (Contrast B with the lack of a primary duct in the original PTHrP-null embryos as shown in Fig. 1E,F) (C) Photomicrograph of an H&E-stained section through the mammary gland of a K14-PTHrP/PTHrP-null neonate. Note that the primary duct extends from the epidermis through the dermis and forms its initial branches (arrows), as occurs in normal mammary development. However, despite the rescue of mammary epithelial development, note the lack of a nipple sheath. Scale bar represents 67 μm in A,B; 17 μm in C.

As expected, the K14-PTHrP/PTHrP-null mice died at birth due to the skeletal abnormalities resulting from the lack of chondrocyte PTHrP expression but, as opposed to the original PTHrP-knockout mice, these mice had mammary glands. As described in the previous sections, by birth, the epithelial duct system in the PTHrP-knockout embryos had completely degenerated. In contrast, as seen in Fig. 6, at birth, the K14-PTHrP/PTHrP-null mice had a well-formed primary duct that extended into the mammary fat pad and formed the expected initial branches. Interestingly, grossly, the primary ducts in the

K14-PTHrP/PTHrP-null neonates often appeared somewhat dilated as compared to normals. On H&E section, one could see that the epithelial duct system in the K14-rescued mice had extended below the upper dermis and, although the primary ducts again often appeared somewhat dilated histologically, they formed normal-appearing secondary ducts within the fatty stroma of the mammary fat pad (see Fig. 6C). Of note, despite the near normal appearance of the ductal tree, there remained no nipple sheath, as was also the case in the PTHrP-null embryos (compare Figs 3E and 6C). Therefore, expression of PTHrP in the embryonic mammary cells of PTHrP-null embryos under the control of the K14 promoter allowed the mammary bud to undergo the primary growth spurt but did not rescue nipple sheath formation.

The double-rescue mice lived to maturity in similar fashion to the col II-PTHrP/PTHrP-null mice. Although the double-rescue mice also lacked nipples, they had a mammary duct system. Fig. 7A demonstrates the fourth and fifth inguinal mammary glands taken from a mature double-rescue female. As can be seen, in these mice the reintroduction of PTHrP via the K14 transgene resulted in the successful completion of the initial round of branching morphogenesis and the appropriate extension of the mammary duct system into the mammary fat pad (Fig. 7A,B). However, the resultant duct system appeared to be that of a sexually immature animal. We had observed that female col II-PTHrP/PTHrP-null mice suffer from a form of hypothalamic hypogonadism (unpublished observations), and we hypothesized that this might have impaired the development of the mammary glands in the double-rescue mice. To address this issue, adult, double-rescue females were treated with subcutaneous estrogen and progesterone for 2 weeks. As shown in Fig. 7C,D, ductal growth in the mammary glands of hormonally treated double-rescue females progressed to the borders of the mammary fat pad and was appropriately branched. Therefore, replacement of PTHrP expression in the developing mammary gland via the K14 promoter was sufficient to support the early morphogenesis of the ductal epithelium and to allow for its subsequent growth and ramification.

DISCUSSION

This report records a series of observations that clearly demonstrate that amino-terminal PTHrP is required for the development of the mammary epithelial duct system in mice. First, col II-PTHrP/PTHrP-null mice (devoid of PTHrP in all tissues except for cartilage) lack all mammary epithelial ducts. Second, in PTHrP-knockout embryos, we found a primary failure of branching morphogenesis during embryonic mammary gland development. Third, deletion of the PTH/PTHrP receptor recapitulated the failure of mammary development seen in the PTHrP-knockout embryos. Finally, reintroduction of PTHrP into mammary epithelial cells via the K14-PTHrP transgene rescued the failure of embryonic mammary development seen in the absence of PTHrP and allowed the subsequent development of the mature mammary duct system within the mammary fat pad.

The formation of the embryonic mammary gland occurs in two steps: first, the formation of the mammary bud and, second, the initiation of branching morphogenesis that leads to the formation of the immature ductal tree (Sakakura, 1987). In PTHrP-knockout embryos, the mammary buds formed appropriately, but they failed to undergo the transition successfully into the initial round of branching growth that leads to the typical immature ductal tree. In the absence of PTHrP, the mammary epithelial structures failed to elongate and/or penetrate into the developing fat pad, remaining in the upper dermis and becoming surrounded by a dense condensation of fibroconnective tissue. The mammary epithelial cells subsequently degenerated; the nipple sheath failed to form, and, by birth, all traces of the mammary epithelial duct system disappeared, explaining the lack of mammary structures in the mature col II-PTHrP/PTHrP-null mice. The exact nature of the epithelial cell degeneration in the PTHrP-knockout embryos remains unclear. PTHrP has been shown to regulate chondrocyte differentiation and apoptosis in the developing growth plate (Weir et al., 1996; Amling et al., 1997; Lee et al., 1996; Vortkamp et al., 1996). However, the loss of the mammary epithelial cells in PTHrP-null embryos did not appear to be associated with either their premature differentiation (as measured by β-casein expression) or apoptosis. Histologically, the stromal condensation around the degenerating ducts in the PTHrP-null mice is reminiscent of the androgen-mediated stromal reaction that leads to the deterioration of the mammary rudiment in male embryos (Sakakura, 1987; Kratochwil and Schwartz, 1976). Despite this similarity, in female knockout embryos, the mammary buds appeared normal through E15, a point at which the mammary buds in normal male littermates are actively degenerating. This asynchrony makes it unlikely that modulation of PTHrP secretion and/or PTH/PTHrP receptor signalling is a central feature of the response of the mammary bud to fetal androgens. However, it remains a possibility that alterations in PTHrP signalling might play some role in the deterioration of the mammary epithelial cells in normal male embryos and we are currently pursuing a series of experiments to test this possibility.

The formation of the embryonic mammary gland is a classic example of inductive development involving epithelial-mesenchymal interactions (Sakakura, 1991; Cunha, 1994). Both the formation of the mammary bud and the initial round of branching morphogenesis appear to be critically dependent on a series of reciprocal and sequential signals exchanged between the mammary epithelium and the dense mammary mesenchyme (Thesleff et al., 1995; Cunha, 1994; Cunha et al., 1995; van Genderen et al., 1994; Weil et al., 1995, Yang et al., 1995). Several experiments have suggested that the presumptive mammary epithelium plays an important role in promoting the condensation and formation of the dense mammary mesenchyme (van Genderen et al., 1994; Kratochwil et al., 1996, Thesleff et al., 1995). However, once formed, the mammary mesenchyme appears to direct the formation of the mammary epithelial duct structure as well as to contribute to mammary epithelial cell cytodifferentiation. For example, heterotypic recombination experiments have demonstrated that mesenchymal cells from the fetal mammary gland can induce nonmammary epithelial cells to form mammary ducts and to make milk proteins (Cunha et al., 1995) and can even induce the formation of mammary bud-like structures from the epidermis of non-mammalian species (Propper, 1973; Propper and Gomot, 1973). Likewise, recent studies have demonstrated

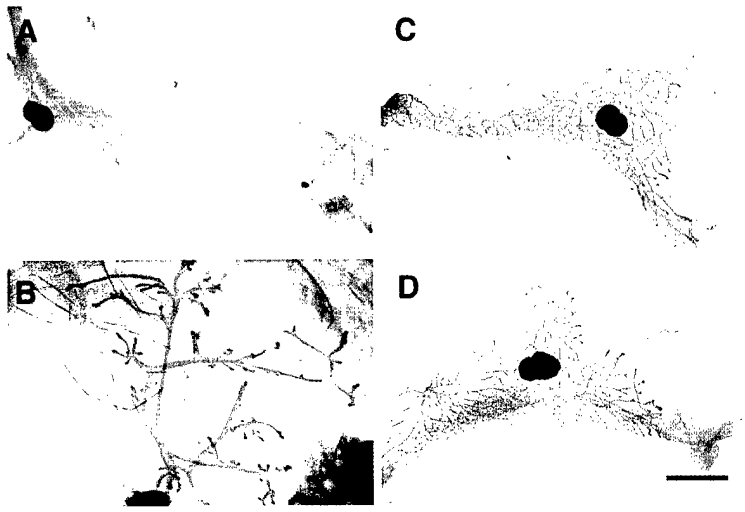

Fig. 7. Mammary development in col II-PTHrP, K14-PTHrP/PTHrP-null (double rescue) mice. (A) Whole-mount analysis of the 4th and 5th inguinal mammary glands taken from a mature double-rescue female mouse (representative of 3 double-rescue mice examined). As one can see, epithelial ducts are present and have penetrated into the fat pad, but the duct appears sexually immature. (B) Higher magnification of 5th inguinal gland shown in A. (C,D) Whole-mount analysis of a mature normal gland (D) and an estrogen- and progesterone-treated double rescue gland (representative of 2 hormonally treated double-rescue mice). As can be seen after 2 weeks treatment of daily 17 β-estradiol (1mg/day) and progesterone (1 ng/day), the double rescue epithelial ducts (C) have grown to fill out the fat pad and appear similar to normals (D). Scale bar represents 5 mm in A and B, and 1 mm in C and D.

that signals derived from mesenchymal cells are important in regulating the overall rate of ductular proliferation as well as the pattern of branching that occurs during the process of branching morphogenesis (Yang et al., 1995; Witty et al., 1995; Phippard et al., 1996). We have demonstrated that, during embryonic mammary development, PTHrP gene expression is limited to the mammary epithelium while PTH/PTHrP receptor gene expression is restricted to the mesenchyme. In the context of the phenotype discussed above, these findings suggest that PTHrP acts as an epithelial message that must be received by the mammary mesenchyme in order for it to support branching growth.

Although mammary development does not appear to be abnormal in the PTHrP knockout embryos until E15-16, we have found that the PTHrP and the PTH/PTHrP receptor genes appear to be expressed in the mammary bud from its formation, at E12, onward. Furthermore, our K14 transgene crossing experiment suggests that PTHrP is largely dispensable before E15. K14 expression does not appear before this point, and therefore the mammary epithelium in the K14-PTHrP/PTHrP-null mice does not produce PTHrP before E15. Despite this delay in PTHrP secretion, as compared to normal mice, K14-PTHrP/PTHrP-null mice successfully initiate branching growth of the mammary ducts. This would imply that the critical period of PTHrP signalling for initiating branching morphogenesis is just before the primary growth spurt at E15-16. However, since nipple sheath development was not rescued in the K14-PTHrP/PTHrP-null mice and since the primary duct did not appear to be completely normal, PTHrP most likely also exerts earlier effects on the mesenchyme. Future study of the effects of PTHrP on mammary mesenchymal cells should help to clarify the details of the temporal requirements for PTHrP signalling during embryonic mammary development.

In summary, we have found that, during embryonic mammary gland development, PTHrP is a necessary participant in the epithelial-mesenchymal interactions leading to the formation of the rudimentary epithelial duct system. Specifically, PTHrP is produced by the mammary epithelium and appears to act on the mesenchyme, allowing it to support the initiation of branching morphogenesis. We have previously reported that the overexpression of PTHrP in mammary myoepithelial cells had dramatic effects on the process of branching morphogenesis during sexual maturation and pregnancy (Wysolmerski et al., 1995), indicating that PTHrP likely plays an important role in the regulation of this process throughout mammary development. There is also growing evidence of the participation of PTHrP in the reciprocal epithelial-mesenchymal interactions that govern epithelial development in sites other than the mammary gland. For example, the pattern of epithelial PTHrP expression and mesenchymal PTH/PTHrP receptor expression seen in the developing mammary gland has been noted in other developing organs (Lee et al., 1995). In addition, col II-PTHrP/PTHrP-null mice have defects in other ectodermally derived organs (skin, teeth and sebaceous glands) that are dependent on epithelial-mesenchymal interactions for their development (unpublished observations). We anticipate that PTHrP will be found to participate in the regulation of mesenchymal cell function during the development of a number of epithelial organs, and it is our hope that further study of the effects of PTHrP during embryonic mammary development will provide a framework for the general understanding of PTHrP's role in regulating mesenchymal function during organogenesis.

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Parathyroid hormone-related protein signaling is necessary for sexual dimorphism during embryonic mammary development

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SUMMARY

Male mice lack mammary glands due to the interaction of circulating androgens with local epithelial-mesenchymal signaling in the developing mammary bud. Mammary epithelial cells induce androgen receptor (AR) within the mammary mesenchyme and, in response to androgens, the mesenchyme condenses around the epithelial bud, destroying it. We show that this process involves apoptosis and that, in the absence of parathyroid hormone-related protein (PTHrP) or its receptor, the PTH/PTHrP receptor (PPR1), it fails due to a lack of mesenchymal AR expression. In addition, the expression of tenascin C, another marker of the mammary mesenchyme, is also dependent on PTHrP. PTHrP expression is initiated on E11 and, within the ventral epidermis, is restricted to the forming mammary epithelial bud. In contrast, PPR1 expression is not limited to the mammary bud, but is found generally within the subepidermal mesenchyme. Finally, transgenic overexpression of PTHrP within the basal epidermis induces AR and tenasin C expression within the ventral dermis, suggesting that ectopic expression of PTHrP can induce the ventral mesenchyme to express mammary mesenchyme markers. We propose that PTHrP expression specifically within the developing epithelial bud acts as a dominant signal participating in cell fate decisions leading to a specialized mammary mesenchyme.

Key words: Androgen receptor, Tenascin C, Epithelial-mesenchymal interaction, Apoptosis, PTH/PTHrP receptor, Mouse

INTRODUCTION

The development of many epithelial organs depends on a series of sequential and reciprocal interactions between epithelial cells and adjacent mesenchymal or stromal cells (Thesleff et al., 1995; Birchmeier and Birchmeier, 1993). The mammary gland is an example of an organ where these epithelial-mesenchymal interactions play a critical role, especially during embryonic development (Sakakura, 1987; Cunha, 1994; Robinson et al., 1999). In mice, mammary development commences with the formation of 5 pairs of epithelial buds located on the ventral surface of the embryo. Each bud begins as a localized thickening of the epidermis first noted on embryonic day 10 (E10, appearance of the vaginal plug = E0), and between E10 and E12-13 this initial placode invaginates into the underlying mesenchyme and the mammary epithelial cells organize themselves into a characteristic "light-bulb" shape (Sakakura, 1987; Robinson et al., 1999). Initially, the mammary mesenchyme is indistinguishable from the ventral dermal mesenchyme, but by the time the mammary epithelial bud is fully formed, it is invested by several layers of mesenchymal cells that are morphologically and functionally distinct from the surrounding dermal mesenchyme (Sakakura, 1987). Recombination experiments have documented that the mammary epithelium and mesenchyme contribute to the formation of each other during mammary bud development and, within the mature mammary bud (through E14-15), each compartment retains the capacity to induce fully the formation of the other (Propper and Gomot, 1967; Heuberger et al., 1982; Cunha et al., 1995).

One of the best-studied aspects of epithelial-mesenchymal interaction during murine mammary development is the androgen-mediated destruction of the mammary bud in males. In male embryos, beginning on E14, the mammary mesenchyme condenses around the neck of the epithelial bud and disrupts the stalk connecting the mammary bud to the overlying epidermis (Turner and Gomez, 1933; Sakakura, 1987). In most strains of mice, the mammary epithelial remnant subsequently degenerates and no nipple is formed, explaining the lack of nipples and mammary glands in adult males (Sakakura, 1987). However, the degree to which the epithelial remnant is destroyed is variable and, in rats, while the stalk is destroyed, there is little degeneration of the remaining epithelium. Several studies have shown that this process occurs as a result of the secretion of androgens by the fetal testes, which act directly on the mammary mesenchyme to trigger its condensation (Raynaud and Frilley, 1947; Raynaud, 1949; Hoshino, 1965; Neuman et al., 1970;

Kratochwil, 1977; Kratochwil and Schwartz, 1977). It has also been demonstrated that the epithelium is a necessary participant in this process, which instructs the mesenchyme to express androgen receptors (Heuberger et al., 1982). Although it is clear that the destruction of the mammary buds by androgens is dependent on a bi-directional flow of information between epithelium and mesenchyme, the identity of the signals exchanged is not known.

One molecule that has recently been implicated in epithelial-mesenchymal interactions at several sites during development is parathyroid hormone-related protein (PTHrP; Wysolmerski and Stewart, 1998). PTHrP was originally discovered as the cause of hypercalcemia in patients with a variety of cancers and it derives its name from a common ancestry shared with parathyroid hormone (PTH; Philbrick et al., 1996). PTHrP also shares the same family of G-protein-coupled receptors with PTH. The prototype of this family is the Type I PTH/PTHrP receptor (PPR1), which appears to subserve the majority of the known functions of PTHrP (Jüppner et al., 1991: Philbrick et al., 1996). During development, PTHrP has been shown to be produced by many developing epithelial structures, while the PPR1 is expressed on adjacent mesenchymal cells, suggesting a role for PTHrP in epithelial-to-mesenchymal signaling, a notion recently confirmed by several PTHrP transgenic and knockout mouse models (Lee et al., 1995; Philbrick et al., 1998; Rubin et al., 1994; Wysolmerski et al., 1994, 1995, 1998).

We have recently shown that PTHrP is necessary for mammary gland development. In the absence of PTHrP or its receptor, there is a failure of the initiation of ductal branching morphogenesis and nipple formation during embryonic mammary development (Wysolmerski et al., 1998). In PTHrP or PPR-1 knockout embryos the mammary bud initially forms normally, but it fails to undergo the primary growth spurt, and the mammary epithelial cells degenerate and disappear before birth (Wysolmerski et al., 1998). Overexpression of PTHrP within the mammary gland also affects branching morphogenesis, leading to an impairment of hormonally stimulated ductal proliferation and side-branching during puberty and early pregnancy (Wysolmerski et al., 1995). Both during embryonic development and during puberty, PTHrP is produced by epithelial cells, while the PPR1 resides on mesenchymal cells during embryonic development and fat pad and periductal stromal cells during puberty (Wysolmerski et al., 1998; Dunbar et al., 1998). Given the patterns of expression of PTHrP and the PPR1 during the early stages of mammary development, and given the requirement for epithelial-mesenchymal interaction in the androgen-mediated destruction of the mammary bud, we initiated a study of PTHrP's possible involvement in this process.

In this report, we document that PTHrP and the PPR1 are necessary for the normal sexual dimorphism seen during murine mammary development. PTHrP is expressed specifically within the epithelial cells of the mammary bud concurrent with its formation, and we identify it to be an epithelial signal responsible for inducing androgen receptor and tenascin C expression within the mammary mesenchyme. Ectopic expression of PTHrP within the fetal epidermis results in the expression of mammary mesenchyme markers in the fetal dermis. These findings suggest that PTHrP participates in regulating the mesenchymal cell fate decisions that result in the formation of a specialized mammary mesenchyme.

MATERIALS AND METHODS

Mouse strains

The disrupted PTHrP and PPR1 alleles were progressively bred onto a CD-1 background to improve litter size and embryo survival, and mice heterozygous for these alleles were mated to produce homozygous PTHrP- and PPR1-null embryos (appearance of vaginal plug=day 0). Wild-type littermates were used as normal controls. Embryos were removed from the uterus and genotyped using the polymerase chain reaction as described previously (Wysolmerski et al., 1998). Keratin 14 (K14) is expressed in specific subsets of epithelial cells, including fetal mammary epithelial cells and basal keratinocytes of the skin, and we have previously documented that the K14 promoter can successfully target PTHrP transgene expression to these cells (Wysolmerski et al., 1998). In the present study, K14-PTHrP embryos were identified as reported previously, and K14-PTHrP/PTHrP-null embryos were produced by mating mice both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null allele with mice heterozygous for the disrupted PTHrP allele (Wysolmerski et al., 1998). All embryos were sexed both by visual inspection of the gonads and by amplification of a 240 bp fragment of the SRY gene by PCR (Wysolmerski et al., 1998).

Histology/immunohistochemistry

After harvesting, embryos were fixed in 4% paraformaldehyde at 4°C for 12 hours. The ventral skin was removed and the intact mammary glands were dissected and embedded in paraffin. Appropriate sections were identified by serial sectioning and hematoxylin and eosin staining, and immunohistochemistry was performed using standard techniques. The androgen receptor antibody is a rabbit polyclonal and was the kind gift of Dr Gail Prins (The University of Illinois at Chicago, Chicago, Illinois). Primary incubations with the androgen receptor antibody were performed at 4°C for 12 hours at a concentration of 0.5 or 1.0 μg/ml and were preceded by boiling of the sections for 30 minutes in 0.01 M citrate buffer pH 6.0. Competition experiments were performed with AR21, which consists of the first 21 amino acids of the androgen receptor and contains the antibody epitopes, and with peptide AR462, which consists of amino acids 462-478 and does not contain the epitopes (peptides courtesy of Dr Prins). The tenascin C antibody is also a rabbit polyclonal antiserum and was the kind gift of Drs Toshimichi Yoshida and Teruyo Sakakura (Mie University, Tsu, Japan). Primary incubations were performed at a concentration of 2.5 or 5.0 μg/ml at room temperature for 1 hour and were preceded by a 10 minute incubation in 0.1% trypsin in 0.1% (w/v) calcium chloride pH 7.8. Primary antibodies were detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and either 3, 3′ diaminobenzidine or TrueBlue™ peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as chromagens. Apoptosis was detected by terminal deoxytransferase labeling (TUNEL assay) utilizing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Indianapolis, IN).

In situ hybridization

In situ hybridization on paraffin sections was performed as previously described (Dunbar et al., 1998; Wysolmerski et al., 1998). Probes were generated from a 349 bp genomic fragment of the mouse PTHrP gene and a 238 bp cDNA fragment of the type I PTH/PTHrP receptor gene (Dunbar et al., 1998; Wysolmerski et al., 1998). Whole-mount in situ hybridization was performed using a protocol kindly provided by Dr Trevor Dale (Phippard et al., 1996). In brief, embryos were harvested, fixed for 2 hours in 4% paraformaldehyde at room temperature, treated with proteinase K (20 μg/ml) for 10-15 minutes

at room temperature and postfixed in 4% paraformaldehyde/0.1% glutaraldehyde for 20 minutes at room temperature. The embryos were then hybridized with digoxigenin-labeled riboprobes for PTHrP and PPR1 generated from the templates described above using the Genius kit (Boehringer Mannheim, Indianapolis, IN). The hybridization buffer consisted of 50% formamide, 1.3× SSC, 5 mM EDTA, 0.2% Tween 20, 0.5% CHAPS and 50 μg/ml yeast RNA and the hybridization was at 70°C overnight. Samples were then washed twice in hybridization buffer for 30 minutes at 70°C, once in 1:1 hybridization buffer:TBST at 70°C for 20 minutes and twice in TBST at room temperature for 30 minutes. Following these washes, the embryos were incubated in blocking solution consisting of 10% sheep serum and 1 mg/ml BSA in TBST for 3 hours at room temperature and then were incubated with anti-digoxigenin antiserum (Genius kit, Boehringer Mannheim, Indianapolis, IN) overnight at 4°C. The color reaction was performed according to the manufacturer's protocol and signals developed between 1 and 2 hours.

RESULTS

PTHrP and the PTH/PTHrP receptor are necessary for sexual dimorphism during murine mammary development

In order to ascertain if PTHrP signaling contributed to the androgen-mediated destruction of the male mammary bud, we examined the gross appearance of the mammary buds in PTHrP- and PPR 1-knockout mice. We performed this analysis on male embryos at E15, a time point at which the destruction of the mammary buds should normally be well advanced (Sakakura, 1987). First, we examined 48 male embryos resulting from crosses between heterozygous PTHrP-null parents. In all 10 wild-type embryos, the mammary buds were either completely absent or consisted of very small remnants. In stark contrast, in each homozygous PTHrP-knockout embryo, all mammary buds were present, well preserved and indistinguishable from those observed in female embryos. There was little evidence of haplotype insufficiency, since only one of 28 heterozygous PTHrP-knockout embryos failed to demonstrate the expected destruction of the mammary buds. We next examined 10 male PPR 1-knockout embryos and found that they uniformly also had the abnormal persistence of mammary buds at E15.

The histological findings in these embryos are shown in Fig. 1. Fig. 1A shows the typical appearance of a wild-type female mammary bud at E15. In contrast, at E15, the wild-type male bud is actively being destroyed (Fig. 1B). There is extensive mesenchymal condensation above the epithelial remnant in the region where the bud stalk appears to be degenerating. The stalk has been interrupted and the epithelial remnant, which is misshapen and degenerating (see TUNEL data below), is no longer connected to the epidermis. However, in PTHrP- and PPR 1-knockout males (Fig. 1C,D), the mammary buds appear similar to those seen in female embryos. In these embryos, there is no mesenchymal cell condensation, and the mammary mesenchyme continues to consist of several layers of cells arrayed concentrically around the epithelial bud. In addition, the epithelial stalk is intact, and the mammary epithelial cells maintain their connection with the epidermis. The mutant male buds persist until E16-E17, at which point they fail to undergo the initial round of branching morphogenesis and instead degenerate, findings identical to that previously described for female PTHrP and PPR-1 knockout mammary rudiments (data not shown) (Wysolmerski et al., 1998).

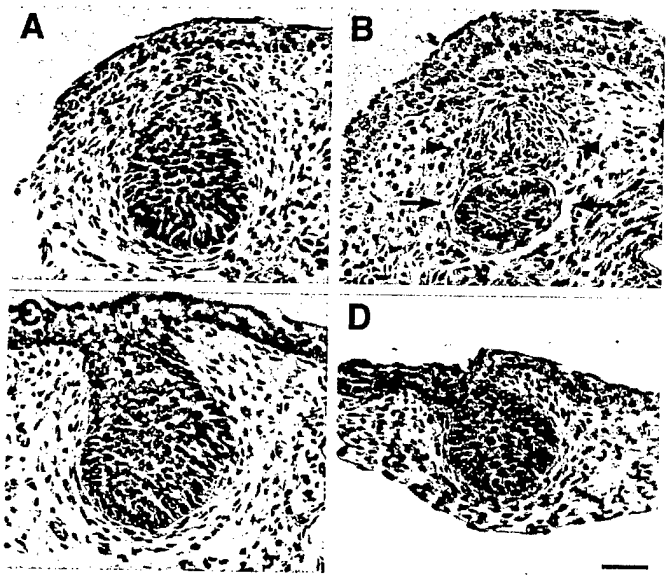

Fig. 1. Failure of the androgen-mediated destruction of the mammary buds in PTHrP- and PPR1-knockout embryos. Hematoxylin- and eosin-stained sections through E15 mammary buds taken from a wild-type female embryo (A), a wild-type male embryo (B), a PTHrP-knockout male embryo (C) and a PPR1-knockout male embryo (D). In the normal male embryo (B), the mesenchymal cells have condensed around the degenerating epithelial stalk (between arrowheads) and the epithelial remnant is misshapen and disconnected from the epidermis (arrows). In contrast, note the lack of mesenchymal condensation and the well preserved epithelial stalks in C and D. Scale bar, 110 μm.

The destruction of the mammary bud in male embryos is an example of programmed cell death (Fig. 2). In the wild-type male bud at E15 (Fig. 2A), there is widespread TUNEL staining in the region of the degenerating epithelial stalk. This appears to involve both the epithelial cells of the stalk and the mesenchymal cells within the androgen-induced condensation. In addition, there is evidence of apoptosis occurring within the epithelial remnant that lies beneath the epidermis. In contrast, in PTHrP-knockout males (Fig. 2B), there is no apoptosis. Similar results were obtained in PPR-1 knockout embryos and, in both strains of knockout mice, the lack of TUNEL-staining was identical to the results obtained with wild-type female embryos (results not shown). Therefore, in the absence of PTHrP or the Type 1 PTH/PTHrP receptor, the pattern of sexual dimorphism normally observed during early mammary development is abolished.

PTHrP and PPR1 are necessary for androgen receptor and tenascin C expression in the dense mammary mesenchyme

The androgen-mediated destruction of the mammary bud is dependent on the presence of functional androgen receptors within the dense mammary mesenchyme, and the expression of these receptors is induced by signals from the mammary epithelium (Heuberger et al., 1982; Sakakura, 1987). The absence of an androgen response in the PTHrP- and PPR1-knockout buds combined with the epithelial expression of

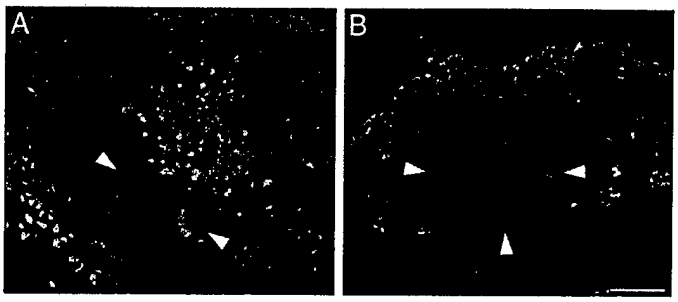

Fig. 2. Programmed cell death in male mammary buds at E15. (A) Results of a TUNEL assay performed on a section through a wild-type male mammary bud. Apoptotic nuclei stain bright green whereas normal nuclei stain pale green. Note the multitude of apoptotic nuclei in the region of the stalk and mesenchymal condensation. Note that there is apoptosis also occurring in the epithelial remnant (between arrowheads). (B) Results of a TUNEL assay performed on a section through a PTHrP-knockout male mammary bud. Note that the mammary bud (outlined by arrowheads) is well preserved and that there is no apoptosis, as demonstrated by the lack of bright green nuclei, in either the epithelial bud or in the mammary mesenchyme. Scale bar, 80 μm.

PTHrP and the mesenchymal expression of the PPR1 (Dunbar et al., 1998; Wysolmerski et al., 1998), led us to posit that PTHrP might be the epithelial signal responsible for the induction of androgen receptor expression within the dense mammary mesenchyme. To investigate this possibility, we examined androgen receptor expression in the mammary buds of wild-type female embryos and male and female PTHrP- and PPR1-knockout embryos at E15 by immunohistochemistry (Fig. 3A-C). In the wild-type bud (Fig. 3B), one can appreciate the intense nuclear staining for androgen receptor in the cells comprising the dense mammary mesenchyme. There is no staining in the general dermal mesenchyme. This pattern of androgen receptor localization is identical to that seen in previous studies using [^{3}H]testosterone autoradiography (Heuberger et al., 1982). In contrast, this staining pattern is absent in the PTHrP- (Fig. 3A) or PPR1-knockout (Fig. 3C) buds. In these glands, there are only occasional nuclei that stain weakly for androgen receptor within the mesenchymal cells closest to the epithelial basement membrane. The absence of androgen receptor staining appears to be specific for the mammary mesenchyme, for androgen receptor staining is normal within the testes of Col II-PTHrP/PTHrP-null (Col II-rescued) mice that lack PTHrP in all tissues except the skeleton (data not shown) (Majdic et al., 1995; Philbrick et al., 1998; Wysolmerski et al., 1998). Furthermore, the development of the Wolffian ducts and the descent of the fetal testes are normal in the absence of either PTHrP or the PPR1, demonstrating an intact androgen response in these tissues (Gilbert, 1994; Grumbach and Conte, 1992).

Androgen receptor expression is one of the characteristics of the mammary mesenchyme that sets it apart from the dermal mesenchyme (Sakakura, 1987), so that the absence of androgen receptor expression within the mammary mesenchyme of PTHrP- and PPR1-knockout embryos suggested that there might be more fundamental defects in the differentiation of these cells. The other classic marker of the specialized mammary mesenchyme is tenascin C (Sakakura, 1987). Therefore, we next examined PTHrP- and PPR1-knockout mammary buds for the expression of this extracellular matrix protein by immunohistochemistry (Fig. 3D-F). The results were similar to those seen with respect to androgen receptor expression. The wild-type epithelial bud (Fig. 3E) was surrounded by a halo of tenascin C within the extracellular matrix of the dense mammary mesenchyme, but not within the general dermal matrix. In contrast, there was no tenascin C expression surrounding the PTHrP- (Fig. 3D) and PPR1- knockout buds (Fig. 3F), suggesting that, in the absence of PTHrP or PPR1, the dense mammary mesenchyme does not differentiate properly. As with androgen receptor expression, there did not appear to be a generalized defect in tenascin C expression, as there was ample tenascin staining in the developing bones of knockout embryos (data not shown) (Erickson and Bourdon, 1989).

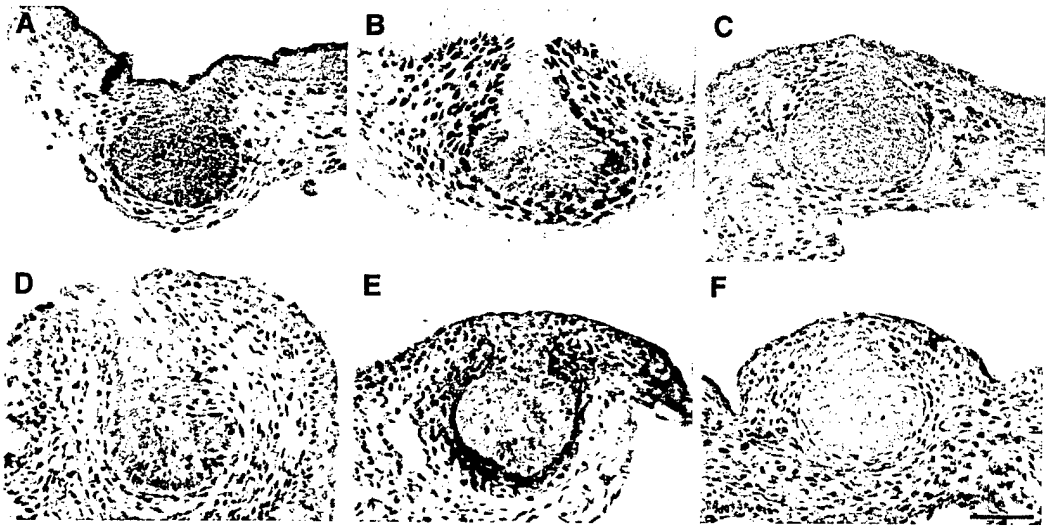

Fig. 3. Androgen receptor and tenascin C staining of E15 mammary buds. (A-C) Sections stained for androgen receptor and (D-F) represent sections stained for tenascin C. (A,D) Sections through male PTHrP-knockout mammary buds. (B,E) Sections through female wild-type buds. (C,F) Sections through male PPR1-knockout mammary buds. Again, note the well-preserved mammary buds in the male knockouts. There is prominent nuclear staining for androgen receptor in the dense mammary mesenchyme of the wild-type bud in B, but little or no androgen receptor staining in the PTHrP-knockout bud in A and the PPR-1-knockout bud in C. Likewise, there is prominent staining for tenascin C in the extracellular matrix surrounding the wild-type bud (E), but an absence of similar staining in the knockouts (D,F). Scale bar, 120 μm.

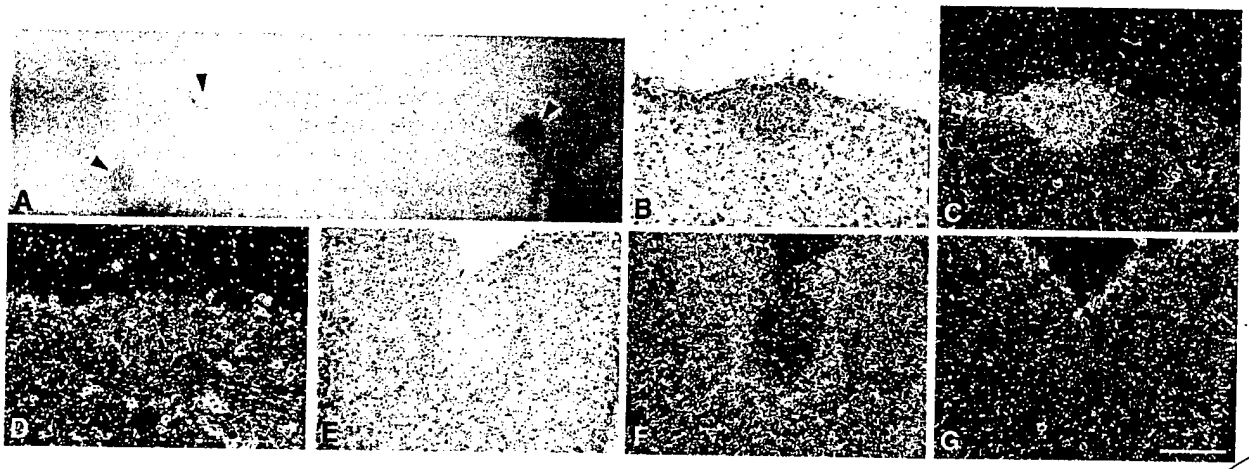

Fig. 4. Initiation of PTHrP expression in developing mammary buds. (A) A representative photograph of whole-mount in situ hybridization experiments using PTHrP antisense probe on a late E11 embryo. The photograph shows a magnified view of the ventral-lateral surface of the embryo. PTHrP expression is not seen within the general epidermis, but is limited to the mammary buds (~~2, 3 and 4~~ arrowheads). (B-D) In situ hybridization for PTHrP on sections through mammary buds taken from E12 embryos. (B,C) Bright-field and dark-field views of the same section, which was hybridized to a PTHrP antisense probes. (D) Section hybridized to a PTHrP sense probe. Note that PTHrP is expressed within the epithelial cells of the mammary bud. (E-G) In situ hybridization for the PPR1 on sections through mammary buds taken from E12 embryos. (E,F) Bright-field and dark-field views of the same section, which was hybridized to a PPR1 antisense probes. (G) Section hybridized to a PPR1 sense probe. Note that the PPR1 is expressed throughout the ventral dermal mesenchyme as well as within the dense mammary mesenchyme. Scale bars, (A) 380 μm; (B-G) 150 μm.

PTHrP is expressed specifically within the forming mammary epithelium

We reasoned that, if PTHrP were to participate in regulating the differentiation of the dense mammary mesenchyme, it should be expressed early during the formation of the mammary bud. In mice, this process is initiated on E10 and is complete by E14-15. We have previously demonstrated that PTHrP is expressed within the mammary epithelium in the fully formed mammary bud (Dunbar et al., 1998; Wysolmerski et al., 1998). To detect the onset of PTHrP expression during the formation of the mammary bud, we performed whole-mount in situ hybridization on wild-type embryos from E10-E12. There was no expression of PTHrP in the ventral epidermis until late on E11, after the mammary buds had already begun to form and, by late E11-E12, there was strong and specific hybridization for PTHrP within the developing mammary buds (Fig. 4A). In situ hybridization on sections through developing mammary buds confirmed these findings, demonstrating that PTHrP was expressed in the mammary epithelial cells invaginating into the underlying mesenchyme (Fig. 4B-D). There was little, if any, expression of PTHrP within the ventral epidermis apart from the mammary buds at these stages. These findings are identical to those obtained by other investigators in whole-mount in situ experiments performed on E13 embryos (K. Lee and G. Segre, personal communication). PPR1 expression was found throughout the ventral mesenchyme both underlying the epidermis and surrounding the mammary buds (Fig. 4E-G).

Re-expression of PTHrP reestablishes sexual dimorphism

PTHrP and PPR1 are both expressed within the embryo as early as the morula stage. Therefore, it is possible that the changes that we observed in the knockout embryos were not

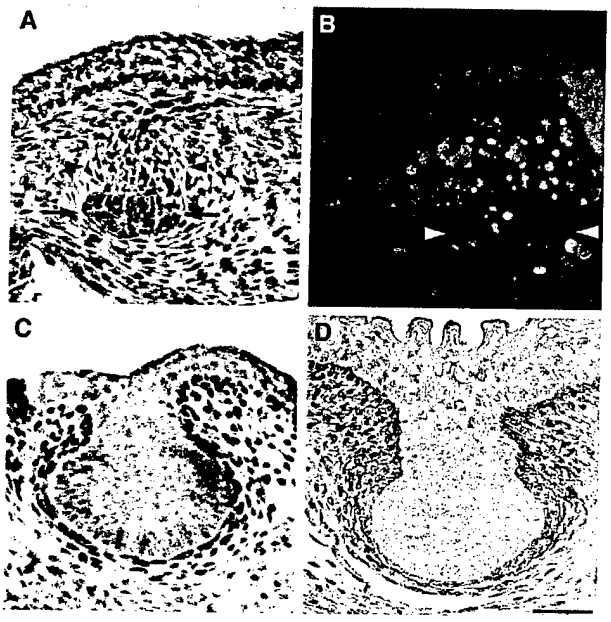

Fig. 5. Return of sexual dimorphism in K14-PTHrP/PTHrP-null embryos. (A) Histology of a mammary bud taken from a male K14-PTHrP/PTHrP-null embryo at E15. Note the typical mesenchymal condensation around the epithelial stalk (between arrowheads) and the degenerating epithelial remnant (between arrows) (compare with Fig. 1B). (B) TUNEL assay performed on a male K14-PTHrP/PTHrP-null embryo at E15. Note the return of the apoptotic response in both the mammary mesenchyme and the epithelial remnant (arrowheads). (C) Androgen receptor staining in a female K14-PTHrP/PTHrP-null embryo at E15. (D) Tenascin C staining in a female K14-PTHrP/PTHrP-null embryo at E15. Note that with the restoration of PTHrP to the mammary epithelium, both molecules are again induced within the mammary mesenchyme. Scale bar, 100 μm.

the result of the loss of PTHrP-signaling from mammary epithelium to mammary mesenchyme during the formation of the mammary bud, but were instead the consequence of earlier changes in mesenchymal patterning (de Stolpe et al., 1993; Behrendtsen et al., 1995). In order to demonstrate a direct link between PTHrP production by the mammary epithelium and mesenchymal cell differentiation, we utilized transgenic mice overexpressing PTHrP under the control of the keratin 14 promoter (K14-PTHrP mice) to restore PTHrP to the mammary epithelium of PTHrP-knockout embryos (Wysolmerski et al., 1998). We have previously shown that this promoter directs transgene expression to the epithelial cells of the fetal mammary gland and have recently observed that a K14-driven β-galactosidase transgene is expressed within the mammary bud as early as E12 (P. R. D. and J. J. W., unpublished results). Therefore, we reasoned that a K14-transgene replacement strategy would be expected to duplicate the normal expression of PTHrP within the mammary bud. The K14-PTHrP transgene was bred onto a homozygous PTHrP-null background, producing embryos (K14-PTHrP/PTHrP-null) that were devoid of PTHrP in all tissues except for the sites of K14 expression (such as mammary epithelial cells). As depicted in Fig. 5, the reintroduction of PTHrP expression within the mammary epithelium resulted in a return of the androgen-mediated destruction of the mammary buds and re-established androgen and tenascin C expression within the dense mammary mesenchyme. On a gross level, at E15, male K14-PTHrP/PTHrP-null embryos possessed only remnants of mammary buds. Histologically, these buds demonstrated the typical features of the androgen-mediated response (Fig. 5A), and TUNEL staining revealed a return of the apoptotic response (Fig. 5B). Androgen receptor (Fig. 5C) and tenascin C (Fig. 5D) staining of female K14-PTHrP/PTHrP-null mammary buds at E15 showed the expected pattern of expression of these markers in the mammary mesenchyme (compare Fig. 5C,D with Fig. 3A,D). These results demonstrate that it is the expression of PTHrP within the epithelium during mammary bud formation that is critical for the normal pattern of sexual dimorphism and suggest that PTHrP signaling from the epithelium to the mesenchyme during early mammary gland development is required for full differentiation of the mammary mesenchyme.

Ectopic expression of PTHrP induces ectopic expression of mammary mesenchyme markers

The mammary phenotypes of the PTHrP- and PPR1-knockout embryos, the specific expression of PTHrP within the mammary epithelial buds and the general expression of the PPR1 within the subepidermal mesenchyme suggested that PTHrP might serve as a dominant signal regulating the fate or course of differentiation of the ventral mesenchyme. We hypothesized that the presence of PTHrP in the mammary bud might lead to the acquisition of the mammary mesenchyme phenotype, while the absence of PTHrP within the general epidermis would be associated with a dermal mesenchyme phenotype. If this were true, ectopic expression of PTHrP within the epidermis might lead to the formation of mammary mesenchyme instead of dermis. In order to test this idea, we again turned to K14-PTHrP transgenic mice. As demonstrated in Fig. 6A, K14 is expressed not only within the mammary epithelium but also within the basal keratinocytes of the fetal epidermis (Kopan and Fuchs, 1989). Therefore, we stained the epidermis of K14-PTHrP transgenic and wild-type littermates for the expression of androgen receptor and tenascin C. In wild-type embryos, we could not detect androgen receptor expression within the ventral dermal mesenchyme but, in the ventral epidermis of K14-PTHrP transgenics, there was clear expression of androgen receptor within the nuclei of the dermal mesenchymal cells closest to the epidermis (Fig. 6B,C). As has been previously reported, there was some tenascin C expression within the basement membrane of the ventral epidermis in wild-type embryos, especially around developing hair follicles (Fig. 6E). However, there was a dramatic accumulation of tenascin C within the basement membrane of the ventral epidermis in K14-PTHrP transgenic embryos as well as an accumulation of tenascin C within the extracellular matrix of the dermal mesenchyme just beneath the epidermis (Fig. 6F). Despite the widespread expression of the PPR1 beneath the epidermis (Fig. 4F,G), only those cells nearest the epidermal source of PTHrP expressed androgen receptor (Fig. 6C) or tenascin C (Fig. 6F), demonstrating that PTHrP acted only over a range of a few cell diameters. Interestingly, these effects also appeared to be limited to the ventral epidermis of the K14-PTHrP embryos. Staining of the dorsal epidermis did not reveal expression of androgen receptor or tenascin C within the dermal mesenchyme (Fig. 6D,G). This was surprising, since both the PPR1 gene and the K14-PTHrP transgene were expressed in both dorsal and ventral epidermis (data not shown). Thus, ectopic overexpression of PTHrP within the ventral epidermis leads to the ectopic expression of androgen receptor and tenascin C within mesenchymal cells that should posses a dermal phenotype, suggesting that, at least on the ventral surface of the embryo, PTHrP may act as a dominant signal to induce the differentiation of the mammary mesenchyme.

DISCUSSION

In this report, we demonstrate that PTHrP, signaling through the PPR1, is essential for the sexual dimorphism in normal murine mammary development. In PTHrP- or the PPR1-knockout embryos, the androgen-mediated destruction of the mammary bud in male embryos fails, due to the absence of androgen receptors in the mammary mesenchyme. In addition, the mammary buds of both types of knockout mice lack tenascin C, an extracellular matrix constituent which is highly expressed within the mammary mesenchyme but not within the dermis (Sakakura, 1987). PTHrP is expressed within the epithelial cells of the forming mammary bud beginning late on E11, however it is not expressed within the mesenchyme or within the epidermis at this stage. In contrast to PTHrP, the PPR1 is expressed within the mesenchyme and its expression is not restricted to the developing mammary bud. Rather, it is expressed both within the mesenchyme surrounding the mammary bud and within the mesenchyme underlying the epidermis. The expression of androgen receptor and tenascin C are directly dependent on PTHrP expression during the formation of the mammary bud and are not a consequence of earlier PTHrP expression, for transgenic expression of PTHrP within the forming mammary epithelial bud in otherwise PTHrP-null (K14-PTHrP/PTHrP-null) embryos leads to the

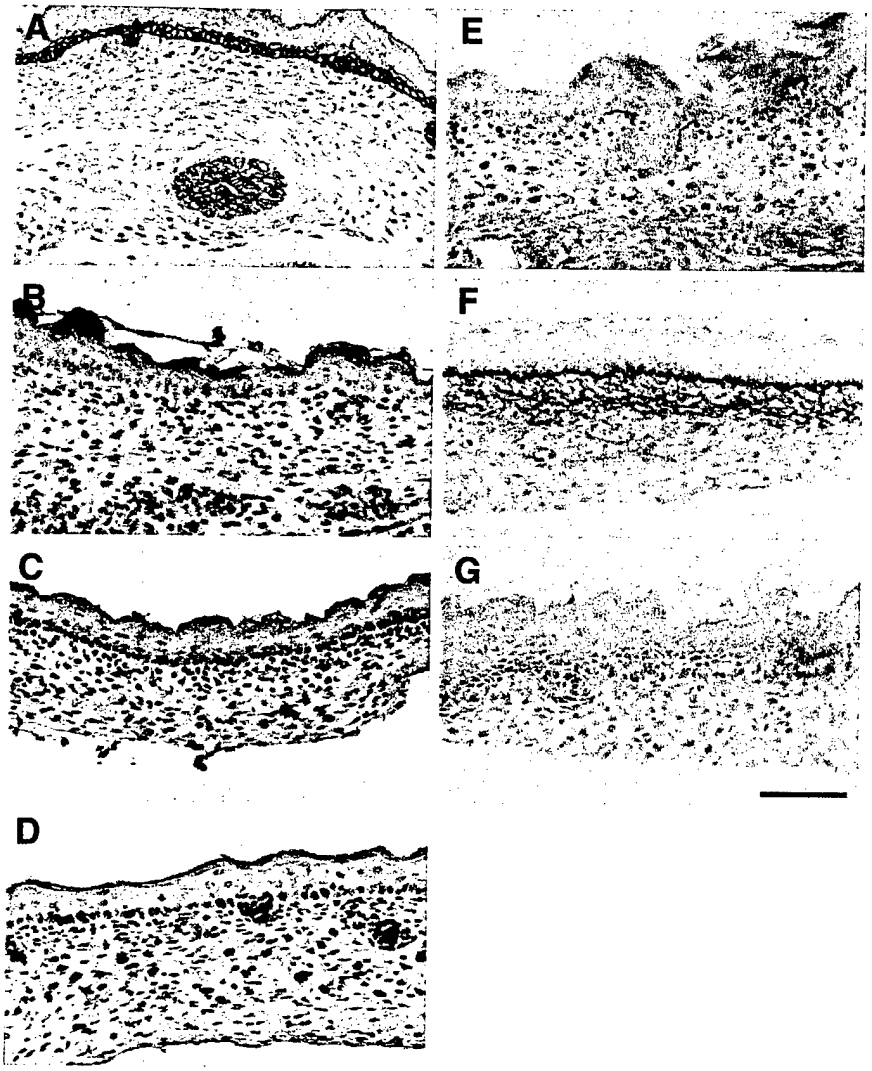

Fig. 6. Ectopic expression of PTHrP in the epidermis induces dermal expression of androgen receptor and tenascin C.
(A) Immunohistochemistry for keratin 14 in the fetal epidermis at E18. Note that K14 is expressed both within the fetal mammary epithelial cells within the mammary duct below the epidermis and within the basal keratinocytes of the epidermis. (B-D) Androgen receptor staining of ventral epidermis from a wild-type embryo at E18 (B), and of ventral (C) and dorsal (D) epidermis from a K14-PTHrP transgenic embryo, also at E18. There is no androgen receptor staining in the wild-type dermis (B), but there is nuclear androgen receptor staining in the dermal cells close to the epidermal basement membrane in the ventral surface of the K14-PTHrP transgenic. However, this is not true for the dorsal aspect of the K14-PTHrP embryos as seen in D. (E-G) A similar pattern is seen for tenascin C. In the ventral surface of wild-type embryos at E18 (E), there is some tenascin expression along the basement membrane, especially in the vicinity of developing hair follicles. However, there is a dramatic upregulation of tenascin within the basement membrane and within the extracellular matrix of the upper dermis on the ventral surface of K14-PTHrP embryos at E18 (F), but not on the dorsal surface of K14-PTHrP transgenic embryos (G). Scale bar, 150 μm.

restoration of the expression of both androgen receptor and tenascin C, and thus the androgen-mediated destruction of the mammary bud. Finally, transgenic expression of PTHrP in the basal epidermis leads to the induction of androgen receptor and tenascin C expression within the ventral dermis, suggesting that ectopic expression of PTHrP may induce an ectopic mammary mesenchyme phenotype.

The expression of androgen receptors and tenascin C has classically distinguished the dense mammary mesenchyme from the surrounding dermal mesenchyme (Sakakura, 1987). It has been known for many years that the mesenchymal expression of both molecules was dependent on short-range inductive tissue interactions with the mammary epithelium, but the nature of the inductive signal(s) sent from epithelium to mesenchyme was not known (Heuberger et al., 1982; Inaguma et al., 1988; Kalembey et al., 1997). Our findings suggest that PTHrP is a vital component in these interactions. However, both of these molecules are expressed elsewhere and their expression is not universally dependent on PTHrP. Likewise, the ability of epidermal overexpression of PTHrP to induce the production of these molecules does not appear to extend to all the dermal mesenchyme, for we did not observe their induction within the dorsal subcutis. Therefore, it is unlikely that PTHrP generally regulates the expression of these molecules. Rather, our hypothesis is that PTHrP, expressed exclusively within the developing epithelial bud, acts as a short-range dominant signal to a receptive ventral mesenchyme to differentiate into dense mammary mesenchyme. This results in the induction of mammary mesenchyme-specific genes (e.g. tenascin and androgen receptor) and the ability of the mesenchyme to support mammary epithelial morphogenesis.

In addition to the failure of androgen responsiveness, the loss of PTHrP-signaling also renders the mammary mesenchyme incapable of supporting the initiation of branching morphogenesis associated with the primary growth spurt on E16 (Dunbar et al., 1998; Wysolmerski et al., 1998). It is unlikely that the loss of either androgen receptor or tenascin C expression explains the inability of the mammary mesenchyme to support the outgrowth of the mammary epithelium in female PTHrP- or PPR1 knockouts because Tfm mice with inactivating mutations of the androgen receptor as well as tenascin C-knockouts both carry out these processes normally (Kratochwil and Schwartz, 1977; Saga et al., 1992). Recently, a series of additional molecules such as BMP-4,

preprotachykinin, Msx 2, Fgf 7, Hoxa9, Hoxb9 and Hoxd9 have been described to be expressed in the mammary mesenchyme (Phippard et al., 1996; Weil et al., 1995; Cunha and Hom, 1996; Robinson et al., 1999; Chen and Capecchi, 1999). However, there is no evidence to date to suggest that the deletion of any of these molecules phenocopies the changes in mammary development noted in the PTHrP- and PPR1-knockouts (Robinson et al., 1999; Chen and Cappechi, 1999). The mammary phenotype of LEF-1-deficient mice remains the closest to that of the PTHrP and PPR1 knockouts (van Genderen et al., 1994; Kratochwil et al., 1996). However, LEF-1 is expressed in the mammary epithelium prior to the onset of PTHrP expression and the failure of mammary development in LEF-1-knockout embryos occurs at an earlier stage than does the failure of mammary development in PTHrP- or PPR1-knockouts. Thus, if LEF-1 and PTHrP are in a common genetic pathway, LEF-1 most likely resides upstream of PTHrP within this pathway (van Genderen et al., 1994; Kratochwil et al., 1996). Except for the expression of androgen receptors and the androgen-mediated destruction of the mammary bud, the nature of the other PTHrP-induced mesenchymal changes that allow the mammary mesenchyme to support morphogenesis remains obscure.

It is also apparent from our results that, although the mammary mesenchyme is dependent on PTHrP for its ability to support morphogenesis, the morphological appearance of the mammary mesenchyme is not dependent on PTHrP. In both PTHrP- and PPR1-knockout embryos, the mammary mesenchyme is histologically indistinguishable from that in normal littermates. The most-likely explanation for these findings is that the condensation or "structural" differentiation of the mammary mesenchyme precedes its functional differentiation (which is dependent on PTHrP). In support of this concept, we have recently observed that syndecan 1, which has been reported to be important to the condensation of tooth mesenchyme, continues to be expressed normally within the mammary mesenchyme of PTHrP and PPR1 knockouts (P. R. D., unpublished observations; Salmivirta et al., 1991; Thesleff et al., 1995). It is likely that there are one or more reciprocal exchanges between the mammary epithelium and the mammary mesenchyme that precede the actions of PTHrP and it will be important to examine the mechanisms leading to activation of PTHrP expression within the developing epithelial bud.

In summary, we have found that PTHrP and the PPR1 are necessary for sexual dimorphism during murine mammary development. PTHrP is an inductive signal from the epithelium to the mesenchyme that is necessary for androgen receptor and tenascin C expression within the mesenchyme. We propose that PTHrP participates in the regulation of mesenchymal cell fate decisions leading to a distinct mammary mesenchyme with the ability to support early mammary morphogenesis. The specific initiation of PTHrP gene expression within the epithelium of the forming mammary bud, the more general expression of the PPR1 within the subepidermal mesenchyme, the ability of ectopic epidermal expression of PTHrP to induce the inappropriate dermal expression of androgen receptor and tenascin C, the short-range nature of this signaling and the inability of the mesenchyme to support morphogenesis in the absence of PTHrP or the PPR1 all support this model. PTHrP has been shown to participate in fetal bone morphogenesis in part by directly regulating the differentiation of proliferating chondrocytes within the growth plate (Chung et al., 1998). In addition, PTHrP has been shown to regulate the morphogenesis of several epithelial organs other than the mammary gland, such as tooth, hair follicles and lung, and, at these sites, it most likely also contributes to the regulation of epithelial-mesenchymal interactions (Philbrick et al., 1998; Rubin et al., 1994; Wysolmerski et al., 1994). The current data suggest that PTHrP regulates epithelial morphogenesis in the fetal mammary gland by regulating mesenchymal cell fate decisions and we anticipate that this will be the case in other organs as well.

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Stromal Cells Are Critical Targets in the Regulation of Mammary Ductal Morphogenesis by Parathyroid Hormone-Related Protein

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Parathyroid hormone-related protein (PTHrP) was originally identified as the tumor product responsible for humoral hypercalcemia of malignancy. It is now known that PTHrP is produced by many normal tissues in which it appears to play a role as a developmental regulatory molecule. PTHrP is a normal product of mammary epithelial cells, and recent experiments in our laboratory have demonstrated that overexpression or underexpression of PTHrP in the murine mammary gland leads to severe disruptions in its development. The nature of these phenotypes suggests that PTHrP acts to modulate branching growth during mammary development by regulating mammary stromal cell function. We now demonstrate that throughout mammary development, during periods of active ductal-branching morphogenesis, PTHrP is produced by epithelial cells, whereas the PTH/PTHrP receptor is expressed on stromal cells. In addition, we show that mammary stromal cells in culture contain specific binding sites for amino terminal PTHrP and respond with an increase in intracellular cAMP. Finally, we demonstrate that the mammary mesenchyme must express the PTH/PTHrP receptor in order to support mammary epithelial cell morphogenesis. These results demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal signaling circuit that is necessary for mammary morphogenesis and that stromal cells are a critical target for PTHrP's action in the mammary gland. © 1998 Academic Press

Key Words: **mammary gland development; branching morphogenesis; mammary mesenchyme; mammary stroma; tissue recombination experiments.**

INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was initially discovered because of its pathogenic role in a common paraneoplastic syndrome known as humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). It derives its name from the fact that its gene and the parathyroid hormone (PTH) gene are both descended from a common ancestor through a process of gene duplication (Broadus and Stewart, 1994). As a result, the two genes share sequence homology and structural characteristics that allow amino-terminal species of PTH and PTHrP to signal through the same receptor, termed the type I PTH/PTHrP receptor (Jüppner *et al.*, 1991). Despite these similarities, these two peptides have evolved to serve very different functions. PTH is made solely by the parathyroid chief cells and is secreted into the systemic circulation where it functions as a classical peptide hormone regulating calcium homeostasis. In contrast, PTHrP is made by a wide variety of cell types, does not circulate, and appears to

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function as a local autocrine or paracrine factor influencing cell growth and differentiation (Broadus and Stewart, 1994).

As noted above, amino-terminal PTHrP signals through a seven-transmembrane-spanning, G-protein-coupled receptor known as the type I PTH/PTHrP receptor (Jüppner *et al.*, 1991). This receptor subserves the calcium-regulating functions of PTH and PTHrP in classical PTH-target organs, but, like PTHrP, is also widely expressed in tissues not involved in calcium homeostasis (Orloff *et al.*, 1989). In these sites the receptor is often expressed in cells in close proximity to those expressing PTHrP, suggesting that it also mediates many of the normal physiologic functions of PTHrP (Lee *et al.*, 1995). However, it is also known that PTHrP is a polyprotein which, through a series of posttranslational processing steps, gives rise to several biologically active peptides not containing the amino terminus (Broadus and Stewart, 1994; Soifer *et al.*, 1992; Wu *et al.*, 1996). These peptides presumably signal through distinct, but as yet unidentified, receptors (Kovacs *et al.*, 1996; Orloff *et al.*, 1996).

PTHrP's role in mature organisms remains unclear, but in recent years evidence has accumulated to suggest that this peptide functions as an important developmental regulatory molecule (Dunbar *et al.*, 1996; Philbrick *et al.*, 1996). In fact, they have been reported to be one of the earliest peptide hormone/receptor pairs to be detected during mouse development and appear to participate in the formation of parietal endoderm (Behrendsten *et al.*, 1995; deStolpe *et al.*, 1993). Because PTHrP-knockout mice suffer from a fatal form of chondrodysplasia (Karaplis *et al.*, 1994), the most widely studied aspect of PTHrP's developmental functions has been its role in regulating chondrocyte differentiation during fetal bone development. In the absence of PTHrP, chondrocytes within the fetal growth plate appear to differentiate too rapidly, and the fetal bones ossify prematurely (Amizuka *et al.*, 1994; Karaplis *et al.*, 1994). In contrast, mice overexpressing PTHrP in chondrocytes are born with a cartilaginous skeleton, resulting from a profound delay in chondrocyte differentiation (Weir *et al.*, 1996). Recent studies have demonstrated that PTHrP acts in a feedback loop with Indian Hedgehog and BMP's, in a pathway involved in controlling the rate at which immature chondrocytes progress through their program of differentiation (Kretzschmar *et al.*, 1997; Lanske *et al.*, 1996; Vortkamp *et al.*, 1996).

Another site in which PTHrP has clearly been shown to have an important developmental role is the mammary gland. Soon after its discovery, PTHrP was noted to be expressed in the pregnant and lactating mammary gland and to be present in large quantities in milk (Budayr *et al.*, 1989; Thiede and Rodan, 1988). Its function during pregnancy and lactation is still unknown but more recent data from transgenic models of PTHrP underexpression and overexpression have shown that it participates in the regulation of ductal branching morphogenesis during embryonic development as well as during sexual maturation and early pregnancy (Wysolmerski *et al.*, 1996; 1998). In the absence of PTHrP or the PTH/PTHrP receptor, mammary epithelial buds form, but fail to initiate ductal branching morphogenesis. Instead, the fetal epithelial cells degenerate and mammary glands do not form (Wysolmerski *et al.*, 1998). Overexpression of PTHrP or PTH within the mammary gland also perturbs ductal branching morphogenesis (Wysolmerski *et al.*, 1996). In this case, an excess of PTHrP results in severe defects in ductular proliferation and side branching during puberty and the inhibition of terminal ductule formation during early pregnancy. Hence, amino-terminal PTHrP, acting through the PTH/PTHrP receptor, appears to contribute to the regulation of ductal branching morphogenesis at several different stages of mammary development.

Like many other epithelial organs, the mammary gland is dependent on the sequential and reciprocal exchange of information between epithelial cells and neighboring mesenchymal cells for its proper morphogenesis (Cunha and Hom, 1996; Sakakura, 1991; Thesleff *et al.*, 1995). These epithelial–mesenchymal interactions are especially critical for the regulation of ductal branching morphogenesis. The studies cited above suggest that PTHrP might act to regulate this process during mammary development by serving as an epithelial signal acting to regulate mammary stromal cell function. In this study, we present data to support such a role for PTHrP. We demonstrate that during periods of active ductal branching morphogenesis, the PTHrP gene is expressed in the mammary epithelium, and the PTH/PTHrP receptor gene is expressed in mammary stroma. In addition, we show that mammary stromal cells bind and respond to amino-terminal PTHrP. Finally, we demonstrate that the presence of the PTH/PTHrP receptor in mammary mesenchyme is critical to the ability of the mesenchymal cells to support the initiation of ductal growth.

MATERIALS AND METHODS

In Situ Hybridization

In situ hybridization was performed on 5-μm paraffin sections as described previously (Wysolmerski *et al.*, 1998). Probes corresponded to a 349-bp genomic fragment of the mouse PTHrP gene and a 238-bp cDNA fragment of the PTH/PTHrP receptor gene (Weir *et al.*, 1996). Sense and antisense probes were generated from linearized fragments using an *in vitro* transcription kit (Promega, Madison, WI) in the presence of [^{35}S]UTP (1000 Ci/mmol, Amersham, Life Science, Arlington Heights, IL). Before hybridization, sections were dewaxed and rehydrated, treated with proteinase K (3 μg/ml in PBS for 17 min at room temperature) and acetylated with 0.25% acetic anhydride in the presence of 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 min. Sections were then rinsed in 2× SSC and incubated for 30 min in 0.66% *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) in 2× SSC, rinsed in 2× SSC, dehydrated in graded alcohol, treated with chloroform for 5 min, rehydrated, and then air-dried. The probes (1.5×10^7 cpm/ml) were then hybridized to the samples for 17 h at 54°C in a humidified chamber. Hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 250 μg/ml tRNA,

100 μg/ml salmon sperm DNA, and 50 mM DTT. After hybridization, sections were rinsed in 1× SSC and washed twice in 2× SSC/50% formamide for 5 min at 52°C, rinsed in 2× SSC, and treated with 30 μg/ml RNase A in 2× SSC at 37°C for 30 min. Following two rinses in 2× SSC, sections were again washed in 2× SSC/50% formamide at 52°C for 5 min, dehydrated through graded ethanol, air-dried, and dipped in a mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development of the emulsion, sections were counterstained with hematoxylin and mounted for microscopic examination.

Preparation of Mammary Stromal Cell Cultures

Mammary epithelial and stromal cells were isolated using a modification of a previously described procedure (Haslam and Levely, 1985). Briefly, the No. 4 inguinal mammary glands were dissected from 11- to 14-day pregnant CD-1 mice, minced with razor blades, and incubated overnight at 37°C in a digestion buffer containing DMEM/F12, 5% FBS, 0.2% dispase grade II, 0.2% collagenase type III, 50 μg/ml gentamycin, 100 units/ml nystatin, and 2.5 μg/ml amphoteracin B. Following digestion, the cells were pelleted by centrifugation at 1500 rpm, washed with DMEM, and then filtered through a 70-μm nitex mesh filter to remove mammary epithelial organoids. To enrich the remaining cells for mammary fibroblasts, we used the differential centrifugation method of Voyles and McGrath (1976). The flow-through following filtration was spun at 80*g* for 30 s to pellet epithelial cells, and the resulting supernatant, containing the mammary fibroblasts, was removed and plated in DMEM medium supplemented with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 units/ml nystatin, 2.5 μg/ml amphoteracin B, and 5 μg/ml each insulin and hydrocortisone. Finally, to remove any remaining epithelial cells, the medium was changed 4 h after plating, and the resulting cultures of mammary stromal cells were grown at 37°C in 5% CO_2 for 5 days.

To assess the purity of the mammary stromal cultures, we performed immunohistochemistry using anti-vimentin and anti-keratin 14 and anti-keratin 8,18 antibodies as stromal cell and epithelial cell markers, respectively. The anti-mouse vimentin antibody is a monoclonal antibody and was purchased from Boehringer Mannheim (Indianapolis). The K14 antibody is an affinity-purified rabbit polyclonal antibody and was a kind gift of Dr. D. Roop (Houston, TX). The anti-keratin 8,18 antibody is a mouse monoclonal and was purchased from Nova-Castra (Burlingame, CA). Immunohistochemistry was performed using standard techniques and primary antibody binding was detected using the Vector Elite avidin–biotin kit (Vector Laboratories, Burlingame, CA) and 3, 3′-diaminobenzidine as a chromagen. Slides were counterstained using hematoxlyn.

RNA Isolation and RNase Protection Analysis

Total RNA was isolated from cells using Trizol reagent (Gibco, Gaithersville, MD). RNase protection analysis was performed as described previously (Daifotis *et al.*, 1992) using 2×10^5 cpm of labeled antisense cRNA probes corresponding to a 349-bp *Avr*II *Pvu*II genomic fragment of the mouse PTHrP gene and a 283-bp *Sau*3a–*Pvu*II fragment of the mouse PTH/PTHrP receptor gene. For an internal standard, 5×10^4 cpm of labeled antisense probe corresponding to a 220-bp *Sau*3a–*Sau*3a fragment of the mouse cyclophilin gene was used.

Receptor Binding Assay

The receptor binding assays were performed as described previously (Orloff *et al.*, 1992) using 30,000 cpm/well of ^{125}I-labeled [Tyr36]hPTHrP-(1–36) NH_2 in a final volume of 0.15 ml/well in 24-well plates for 4 h at 4°C in the presence or absence of increasing concentrations of competing unlabeled PTHrP(1–36).

cAMP Assay

Cells were grown to confluence in 12-well plates at 37°C in 5% CO_2. Prior to the experiment, the cells were washed once with serum-free medium containing 0.1% BSA and then incubated with serum-free medium/0.1% BSA with or without PTHrP (1–36) at various concentrations and for various time periods. The medium was then aspirated, and the cells were treated with ice cold 90% *n*-propanol for 24 h at −70°C. The samples were then lyophilized, and intracellular cAMP content was measured using a commercially available RIA (Biomedical Technologies, Stoughton, MA).

Tissue Recombination Experiments

Mammary gland rudiments were dissected from E13 PTH/PTHrP receptor knockout (ko) and wild-type (wt) embryos which were identified by their genotype as determined by PCR as described previously (Wysolmerski *et al.*, 1998). The mammary rudiments were incubated for 1.5 h in 1% Bacto-trypsin in calcium–magnesium-free Hanks' salt solution at 4°C. Following neutralization of the enzyme with 10% fetal bovine serum in DMEM, the epithelium and mesenchyme were teased apart with watchmakers forceps. For PTH/PTHrP receptor knockout embryos, the four possible tissue recombinants were prepared with mammary epithelium (MGE) and mammary mesenchyme (MGM) from wt and ko mice: wt-MGM + wt-MGE, wt-MGM + ko-MGE, ko-MGM + ko-MGE, and ko-MGM + wt-MGE (Cunha *et al.*, 1995). All tissue recombinants were transplanted beneath the renal capsule of female athymic nude mouse hosts (see web site http://mammary.nih.gov/tools/Cunha001/index.html for technical details). Following 1 month of growth, the grafts were harvested for histological analysis (Cunha *et al.*, 1995).

RESULTS

Expression of PTHrP and the PTH/PTHrP Receptor during Ductal Morphogenesis

To begin to study the mechanisms by which PTHrP and the PTH/PTHrP receptor regulate ductal growth and branching morphogenesis, we examined their temporal and spatial patterns of expression during fetal life, during sexual maturation, and during early to mid-pregnancy, three periods of active ductal growth during mammary development. We first sought to determine the temporal pattern of PTHrP and PTH/PTHrP receptor mRNA expression in the whole mammary gland by RNase protection analysis. Due to the small size of the mammary glands, this analysis was not possible for fetal time points and, therefore, we initiated these studies by examining the temporal pattern of PTHrP and PTH/PTHrP receptor expression in the preadolescent gland (3-week-old virgin), during puberty (6-week-old vir-

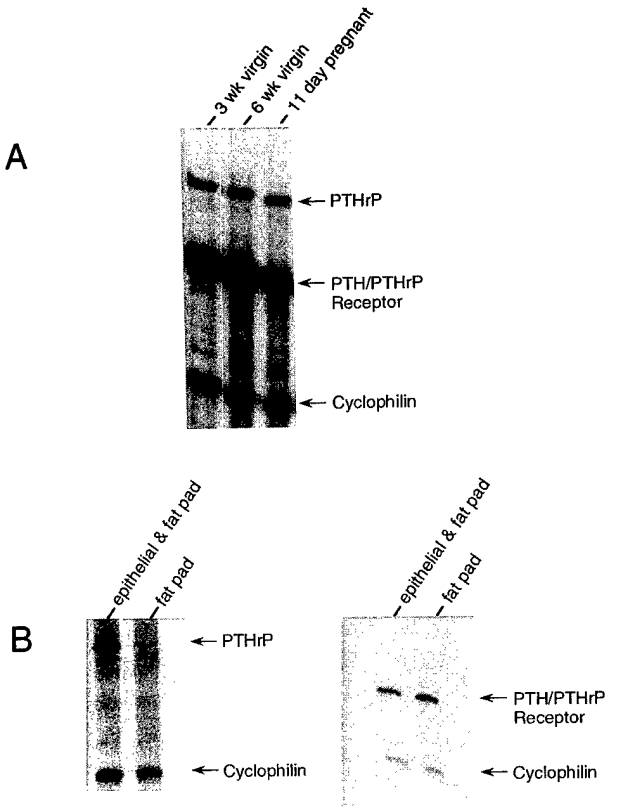

FIG. 1. (A) Analysis of PTH and PTH/PTHrP receptor RNA in the mammary gland during preadolescence, sexual maturation, and pregnancy. 50 μg of total cellular RNA prepared from mammary tissue from preadolescent (3-week-old virgin), adolescent (6-week-old virgin), and pregnant (11 days postcoitus) mice was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. The murine cyclophilin RNA was included as a loading control. Note that both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. (B) Analysis of PTHrP and PTH/PTHrP receptor mRNA expression in proximal and distal segments of preadolescent mammary glands. Mammary glands from 3-week-old virgin mice were dissected and separated into proximal and distal segments. Whole mount analysis confirmed that the proximal segment contained both epithelial and stromal components, while the distal segment contained only stroma (data not shown). 50 μg of total cellular RNA prepared from either the proximal component (epithelial + fat pad) or the distal component (fat pad) was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that the proximal component with both epithelial and stromal cells contains both PTHrP and the PTH/PTHrP receptor mRNA, but the distal component, with stromal cells alone, contains only the PTH/PTHrP receptor.

gin), and during early to mid-pregnancy (11 days postcoitus). As shown in Fig. 1A, both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. In addition, despite the dramatic changes in cellular composition of the mammary gland at these different time points, whole-gland levels of PTHrP and PTH/PTHrP receptor mRNA expression remained relatively constant.

Our prior studies had suggested that, during embryonic mammary development, PTHrP mRNA is expressed in mammary epithelial cells and PTH/PTHrP receptor mRNA is expressed in the mammary mesenchyme (Wysolmerski *et al.*, 1998). To determine if this pattern was also present during the later stages of ductal morphogenesis, we first took advantage of the directional growth of mammary epithelial ducts during puberty. Prior to the initiation of adolescence the mammary ducts occupy only a small portion of one end of the mammary fat pad and, in response to the hormonal stimulation of puberty, they grow toward the opposite end of the mammary fat pad until they completely fill out this stromal compartment. As a result of this growth pattern, at the initiation of puberty, one can divide the murine mammary gland into a proximal segment that contains both epithelial and stromal components and a distal segment that contains only stroma. As shown in Fig. 1B, the proximal component with both epithelial and stromal cells, contains both PTHrP and PTH/PTHrP receptor mRNA, but the distal component, that is stroma alone, contains only PTH/PTHrP receptor mRNA. These data suggest that PTHrP mRNA is expressed in the mammary epithelium, and that PTH/PTHrP receptor mRNA is expressed within the fat pad stroma.

To examine this possibility directly, we next determined the spatial localization of PTHrP and PTH/PTHrP receptor mRNA expression by *in situ* hybridization during fetal life, during adolescence and during early pregnancy. As shown in Fig. 2, the PTHrP gene was expressed in epithelial cells during periods of mammary ductal growth. At E12, we found PTHrP expression to be very intense in the epithelial cells of the embryonic mammary bud, especially in the cells located peripherally, adjacent to the basement membrane (Figs. 2A and 2B). At E18, at a time when the mammary bud is elongating and initiating ductal branching morphogenesis, PTHrP expression continued to be intense and was localized to mammary epithelial cells (Figs. 2C and 2D). Once again, expression of the PTHrP gene was most obvious in epithelial cells located on the outer most layer of the developing mammary ducts. These results are in agreement with our previous findings that demonstrated PTHrP mRNA expression in epithelial cells of the embryonic mammary bud at E16 (Wysolmerski *et al.*, 1998).

In the postnatal mammary gland (Figs. 2E through 2M), PTHrP mRNA expression continued to be localized to epithelial cells. However, overall, expression appeared to be less intense than during fetal development and it appeared to be restricted to epithelial cells located within terminal end buds. End buds are specialized structures that form at the leading edge of growing ducts, and they serve as the sites of active cellular proliferation and differentiation during phases of ductular proliferation (Daniel and Silberstein, 1987). As seen in Figs. 2E–2I, we found that, during puberty, PTHrP mRNA was present in the epithelial cells of end buds (Figs. 2E through 2G), but was undetectable in

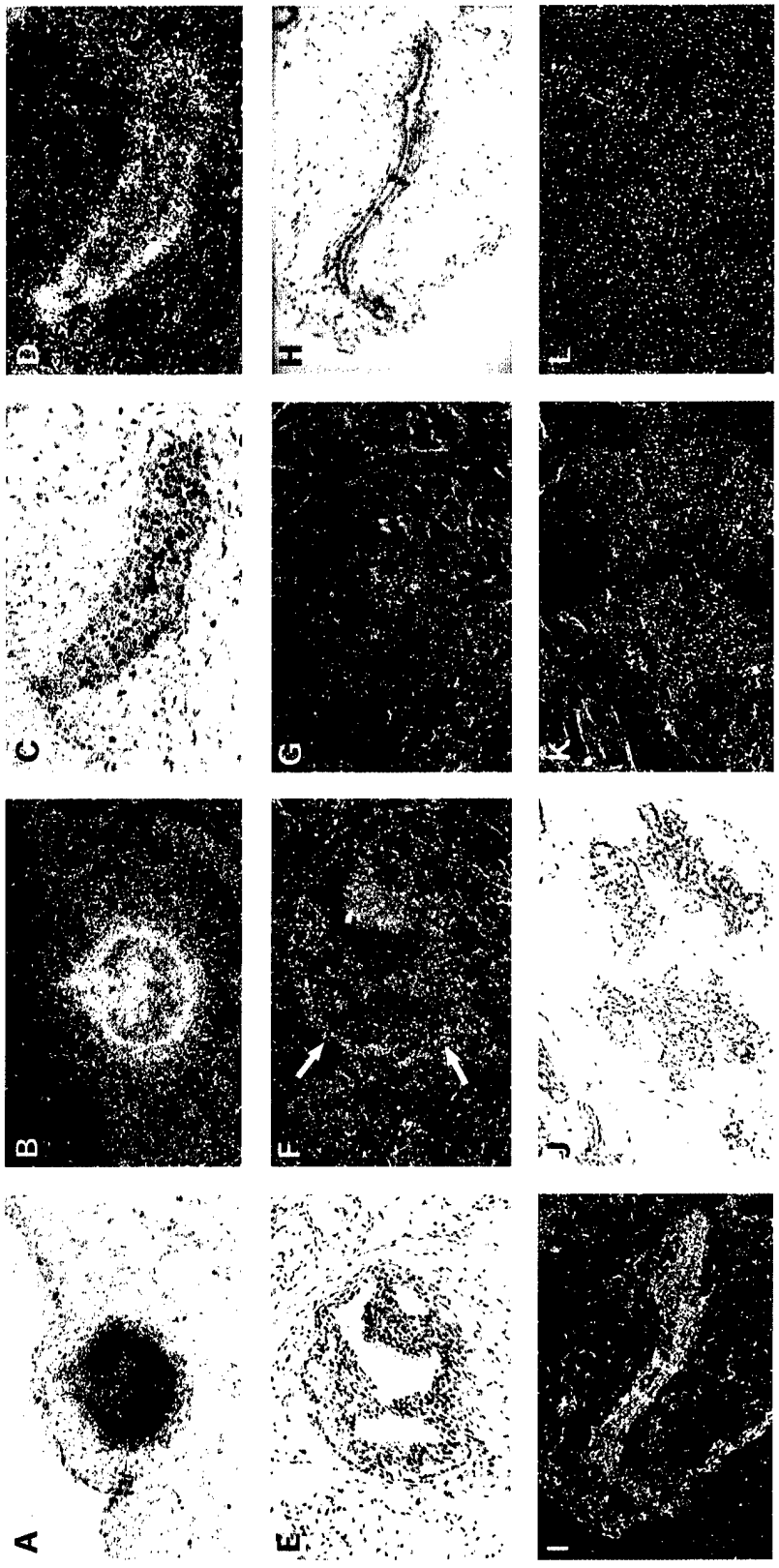

FIG. 2. Localization of PTHrP mRNA expression in mammary glands of embryonic, adolescent, and pregnant mice. (A–D) *In situ* hybridization for PTHrP mRNA in embryonic mammary rudiments at E12 (A and B) and E18 (C and D). A and C represent brightfield images and B and D represent darkfield images, respectively, of the same sections hybridized with antisense probe. (E–I) *In situ* hybridization for PTHrP mRNA in the adolescent mammary gland. E and F represent brightfield and darkfield images, respectively, of a section through an end bud of mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H and I represent brightfield and darkfield images, respectively, of a section through a mature duct of an adolescent mammary gland hybridized with an antisense probe. (J–M) *In situ* hybridization for PTHrP mRNA in the pregnant mammary gland. J and K represent brightfield and darkfield images, respectively, through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days postcoitus) mouse hybridized with an antisense probe. L and M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

epithelial cells of mature ducts (Figs. 2H and 2I). Specifically, the PTHrP mRNA signal appeared to localize to the peripheral, or cap cells of the end buds, a pattern similar to the peripheral location of the PTHrP signal seen during fetal life. During early pregnancy, there appeared to be a very low level of PTHrP mRNA expression within the epithelial cells of developing lobuloalveolar units (Figs. 2J through 2M) but, similar to puberty, we could not detect PTHrP mRNA in mature mammary ducts (data not shown). However, we did detect a similar pattern of epithelial PTHrP expression in the occasional end bud we observed at the periphery of early pregnant glands (data not shown). Therefore, it appears that similar to embryonic life, in the postnatal mammary gland, the major site of PTHrP expression is within epithelial cells. During puberty the PTHrP gene is expressed most prominently in end buds, and during early pregnancy there appears to be a low level of PTHrP expression within the developing lobuloalveolar units.

In contrast to the epithelial pattern of PTHrP mRNA expression, PTH/PTHrP receptor mRNA appeared to be expressed in mammary stromal cells (Fig. 3). In the embryonic mammary gland, at E12, PTH/PTHrP receptor mRNA was expressed throughout the ventral mesenchyme, including the dense mammary mesenchyme (Figs. 3A and 3B). At E18, when the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in stromal cells enveloping the growing mammary ducts (Figs. 3C and 3D). During puberty, PTH/PTHrP receptor mRNA was expressed at a low level throughout the mammary stroma, but the most prominent PTH/PTHrP receptor expression was in stromal cells immediately surrounding terminal end buds (Figs. 3E through 3G). This expression appeared to be most intense at the neck regions of the end buds and decreased rapidly along the more mature portions of the duct so that the majority of the periductal stroma demonstrated a level of receptor mRNA expression indistinguishable or just above the background expression of the fat pad stroma (Figs. 3H and 3I). During early to mid-pregnancy PTH/PTHrP receptor mRNA also appeared to be expressed at a low level throughout the fat pad stroma both within the periductal stroma and surrounding the developing lobuloalveolar units (Figs. 3J through 3M). However, the signal intensity was very low in the pregnant tissue, and there was not striking pattern of hybridization such as that seen during puberty. The exception to this diffuse low level of hybridization was in the occasional gland with a few remaining end buds during early pregnancy, where there was a strong signal within the stroma surrounding the end buds (not shown).

Together, these studies demonstrate that during active mammary ductular branching morphogenesis, PTHrP is expressed by epithelial cells, and its receptor is expressed by surrounding mesenchymal cells. In addition, it appears that in the postnatal mammary gland, expression of the PTHrP and the PTH/PTHrP receptor gene is most intense in terminal end buds, regions of active proliferation and ductal morphogenesis during puberty.

Mammary Stromal Cells Express Functional PTH/PTHrP Receptors

Our *in situ* hybridization results demonstrate that the PTH/PTHrP receptor is expressed in mammary stromal cells and, therefore, suggest that these cells are a target for PTHrP's action in the mammary gland. To test this hypothesis, we prepared primary cultures of both epithelial and stromal cells from adult female mammary glands using previously described protocols (Haslam and Levely, 1985; Voyels and McGrath, 1976) and characterized their composition by immunocytochemistry, using anti-vimentin and anti-keratin 14 and anti-keratin 8, 18 antibodies to identify fibroblasts and epithelial cells, respectively. As defined by the expression of vimentin and the lack of keratin expression, we were able to consistently prepare primary cultures of mouse mammary stromal cells that were 90–95% pure, a level of stromal cell enrichment comparable to that of previous reports (data not shown, Haslam and Levely, 1985).

To determine whether stromal cells in culture continued to express PTH/PTHrP receptor mRNA, total RNA was prepared from our stromal cell cultures and from freshly isolated mammary epithelial organoids and assayed for steady-state levels of both PTHrP and PTH/PTHrP receptor mRNA by RNase protection analysis. As shown in Fig. 4, mammary stromal cells in culture contained PTH/PTHrP receptor mRNA, but not PTHrP mRNA. In contrast, mammary epithelial cells in freshly isolated organoids contained PTHrP mRNA but no PTH/PTHrP receptor mRNA. These results are identical to the results from our *in situ* analysis and lend further support to the epithelial–mesenchymal pattern of expression of PTHrP and the PTH/PTHrP receptor in the mammary gland.

We next examined mammary stromal cells for specific binding sites for amino-terminal PTHrP. For this purpose, receptor binding assays were performed on intact cells, using ^{125}I-labeled PTHrP(1–36) as a ligand. These experiments documented specific binding of ^{125}I-labeled PTHrP-(1–36) amide to mammary stromal cells with an apparent K_d of 8.9 ± 1.4 nM. Binding was specific, as it was effectively competed with unlabeled PTHrP (1–36) amide (Fig. 5). The number of binding sites per cell, as determined by Scatchard analysis, was calculated to be 126,000 ± 13,000. In addition, treatment of mammary stromal cells with PTHrP(1–36) caused a increase in intracellular cAMP over basal (Fig. 6). This cAMP response peaked at 2 min, as shown in Fig. 6a, and showed a dose-dependent increase in cAMP accumulation with maximal responses occurring with 10^{-6} and 10^{-7} M PTHrP (Fig. 6b). Together, these results indicate that mouse mammary stromal cells express the PTH/PTHrP receptor both *in vivo* and *in vitro*, and cultured stromal cells display high-affinity binding sites for amino-terminal PTHrP at their cell surface and respond to PTHrP with an increase in intracellular cAMP.

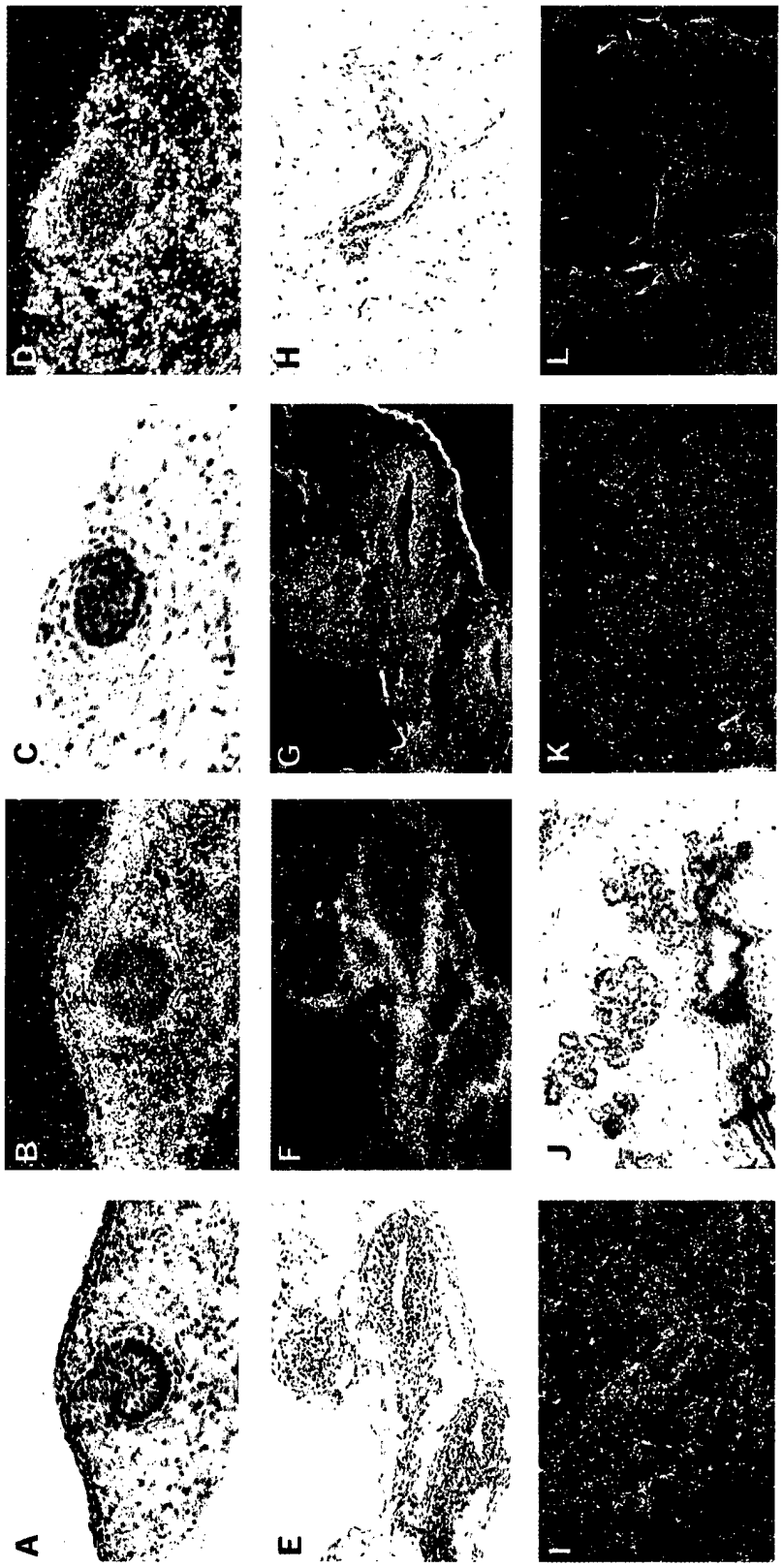

FIG. 3. Localization of PTH/PTHrP receptor mRNA during mammary development. *In situ* hybridization for PTH/PTHrP receptor mRNA in embryonic mammary rudiments at E12 (A and B) and E18 (C and D). A and C represent brightfield images and B and D represent darkfield images of the same sections hybridized with antisense probe. (E–I) *In situ* hybridization for PTH/PTHrP receptor mRNA in the adolescent mammary gland. E and F represent brightfield and darkfield images, respectively, of a section through an end bud of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H and I represent brightfield and darkfield images, respectively, of a section through a mature duct of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. (J–M) *In situ* hybridization for PTH/PTHrP receptor mRNA in the pregnant mammary gland. J and K represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days postcoitus) mouse hybridized with an antisense probe. L and M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

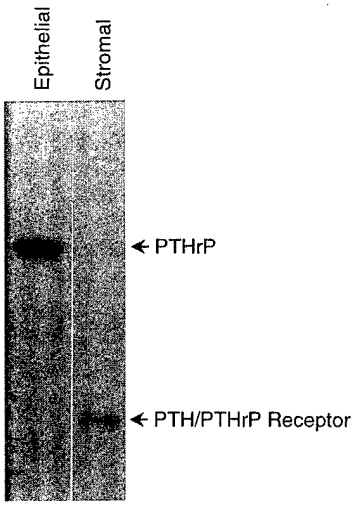

FIG. 4. Analysis of PTHrP and PTH/PTHrP receptor expression in freshly isolated mammary epithelial cells and mammary stromal cells in culture. 50 μg of total cellular RNA from freshly isolated mammary organoids and cultured mammary stromal cells was assayed for both PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that mammary stromal cells in culture express the PTH/PTHrP receptor but not PTHrP mRNA, whereas mammary epithelial cells express PTHrP mRNA but not PTH/PTHrP receptor mRNA.

The Mammary Mesenchyme Must Express the PTH/PTHrP Receptor to Support the Outgrowth of the Mammary Epithelium

We have previously reported that PTHrP and the PTH/PTHrP receptor are required for the outgrowth of the mammary epithelium during embryonic development. In mice lacking either PTHrP or the PTH/PTHrP receptor, mammary buds form but subsequent mammary development fails. In the absence of PTHrP or its receptor, mammary epithelial cells fail to undergo the initial stage of branching morphogenesis known as the primary growth spurt and, instead, degenerate and die (Wysolmerski *et al.*, 1998). These data, together with the expression patterns of PTHrP and the PTH/PTHrP receptor and the presence of functional PTH/PTHrP receptors in mammary stromal cells, as described above, suggest that epithelial-derived PTHrP, acting through stromal PTH/PTHrP receptors, plays an important role in regulating ductal morphogenesis during fetal life and perhaps also at later stages of development (Wysolmerski *et al.*, 1996; 1998). To test this hypothesis directly, we performed a series of tissue recombination and transplantation experiments using mammary epithelial buds and mammary mesenchyme from wild-type and PTH/PTHrP receptor-knockout embryos. In these experiments, knockout and wild-type mammary epithelial buds and mammary mesenchyme were recombined in the four possible combinations (see Table 1) and grown under the kidney capsule of recipient females. We reasoned that if the above hypothesis was valid, and PTHrP and the PTH/PTHrP receptor do represent an epithelial–mesenchymal signaling circuit, the PTH/PTHrP receptor-null phenotype would be expected to segregate with mesenchymal tissue. That is, receptor-knockout mesenchyme should be unable to support the outgrowth of either receptor-knockout or normal epithelial buds, but receptor-knockout epithelium should be able to form ducts when combined with normal mesenchyme.

Table 1 and Fig. 7 summarize the results of these experiments. As expected, wild-type epithelial buds paired with wild-type mammary mesenchyme (wt-MGE + wt-MGM) consistently gave rise to a series of branched epithelial ducts contained within a fatty stroma (see Fig. 7A). In contrast, ductal outgrowth was never detected when PTH/PTHrP receptor-knockout epithelial buds were paired with PTH/PTHrP receptor-knockout mesencluyme (ko-MGE + ko-MGM, see Fig. 7B). These transplants gave rise to a fatty stroma that was devoid of mammary epithelial cells, reproducing the phenotype of the PTH/PTHrP receptor-knockout embryos. Recombinations consisting of PTH/PTHrP receptor-knockout epithelium paired with wild-type mesenchyme (ko-MGE + wt-MGM) uniformly gave rise to branched epithelial ducts within a fatty stroma (Fig. 7C). However, although the receptor-knockout epithelial buds consistently grew out and formed a rudimentary branching ductal structure, the growth of the resulting ducts appeared stunted compared with the ducts produced by wild-type epithelial buds paired with wild-type mesenchyme. Nonetheless, the PTH/PTHrP receptor-knockout epithelial cells survived and had the capacity to initiate branching morpho-

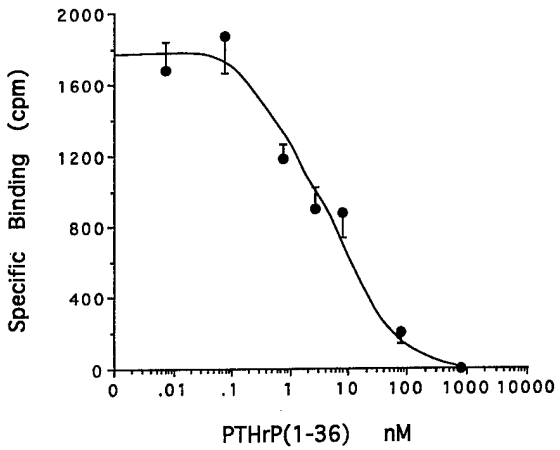

FIG. 5. Binding of PTHrP(1–36) to mammary stromal cells in culture. Receptor binding assays were performed with ^{125}I-labeled PTHrP(1–36) amide as a ligand for 4 h at 4°C with increasing concentrations of unlabeled PTHrP(1–36) as competitor. The data represent the mean ± SE. A representative of three independent experiments is shown.

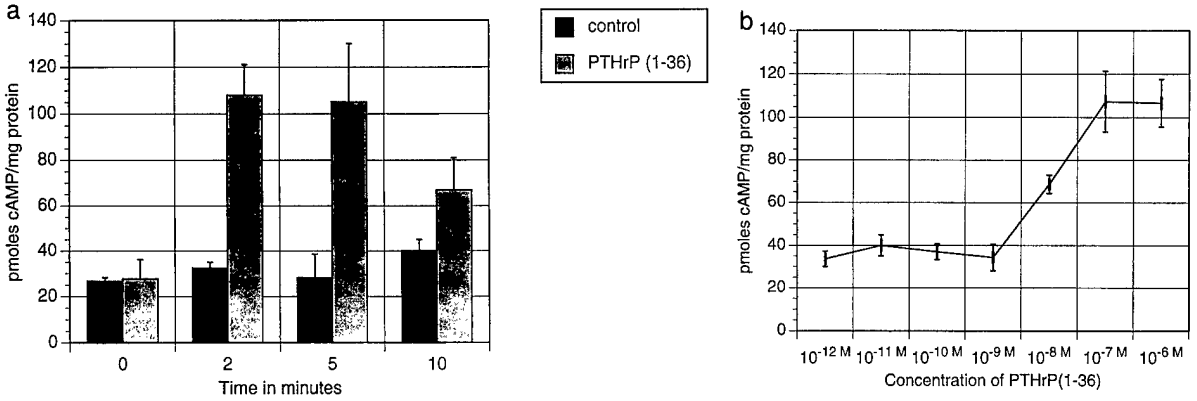

FIG. 6. cAMP response of mammary stromal cells in culture in response to PTHrP(1–36). (a) Time course of cAMP accumulation in primary cultures of mouse mammary stromal cells following treatment with PTHrP(1–36). Mammary stromal cells were incubated for the indicated times at 37°C in serum-free medium with or without 10^{-7} M PTHrP(1–36), and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ± SE for duplicate samples from three separate experiments. (b) Dose-dependent effects of PTHrP(1–36) on cAMP accumulation in mammary stromal cells. Mammary stromal cells were treated with varying concentrations of PTHrP(1–36) for 2 min in serum-free medium, and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ± SE of three experiments each run in duplicate.

genesis when paired with normal mesenchyme. As expected, similar to the results seen with knockout buds paired with knockout mesenchyme, all recombinants composed of wild-type epithelial buds paired with receptor-knockout mesenchyme (wt-MGE + ko-MGM) lacked any evidence of epithelial ductal outgrowth and consisted of fatty stroma alone (see Fig. 7D), suggesting that PTH/PTHrP receptor-knockout mesenchyme was unable to support the survival or morphogenesis of normal epithelial cells in this transplant system. These results demonstrate that the defects in mammary epithelial cell morphogenesis and survival seen in the PTH/PTHrP receptor null mice segregate with mesenchymal tissue and suggest that the mesenchyme is a critical target for the actions of PTHrP during mammary ductal morphogenesis.

DISCUSSION

Mammary gland morphogenesis is dependent on the interplay of systemic endocrine signals and short-ranged epithelial–mesenchymal interactions (Cunha, 1994; Sakakura, 1991). Although the morphological and endocrine aspects of mammary development have been well defined, the paracrine molecules and signaling mechanisms that are influenced by systemic hormones and that regulate mammary epithelial–mesenchymal interactions have only recently begun to be understood (Friedmann and Daniel, 1996; Kratochwil *et al.*, 1996; Phippard *et al.*, 1996; Hennighausen and Robinson, 1998). Mammary ductal growth during puberty is strictly dependent upon estrogens such as estradiol signaling through the estrogen receptor-α, a conclusion derived from the analysis of estrogen receptor-knockout (ERKO) mice (Korach, 1994). In addition, recent analysis of ERKO/wild-type tissue recombinants have shown that stromal estrogen receptors are especially important for the effects of estrogens on ductal growth and branching (Cunha *et al.*, 1997). Several stromal cell factors (some of which are estrogen-responsive) have now been identified that appear to regulate epithelial cell morphogenesis and differentiation (Alexander *et al.*, 1996; Jones *et al.*, 1996; Pollard and Henninghausen, 1994; Sympson *et al.*, 1994; Weil *et al.*, 1995; Witty *et al.*, 1995; Yang *et al.*, 1995). However, less is known about epithelial signals that are involved in regulating stromal cell function during mammary development. Prior experiments in transgenic mice have suggested that amino-terminal PTHrP, acting through the PTH/PTHrP receptor, might play a role in the epithelial–mesenchymal interactions that govern mammary ductal morphogenesis. In this report we provide a series of observations lending further support to the notion that PTHrP is an epithelial signal received by the mammary mesenchyme that is critical for the mesenchyme's ability

TABLE 1
Summary of Tissue Recombination Experiments

Mesenchyme	Epithelium	*n*	Stroma only	Ducts
wt	wt	4	0	4
Receptor-ko	Receptor-ko	4	4	0
wt	Receptor-ko	2	0	2
Receptor-ko	wt	3	3	0

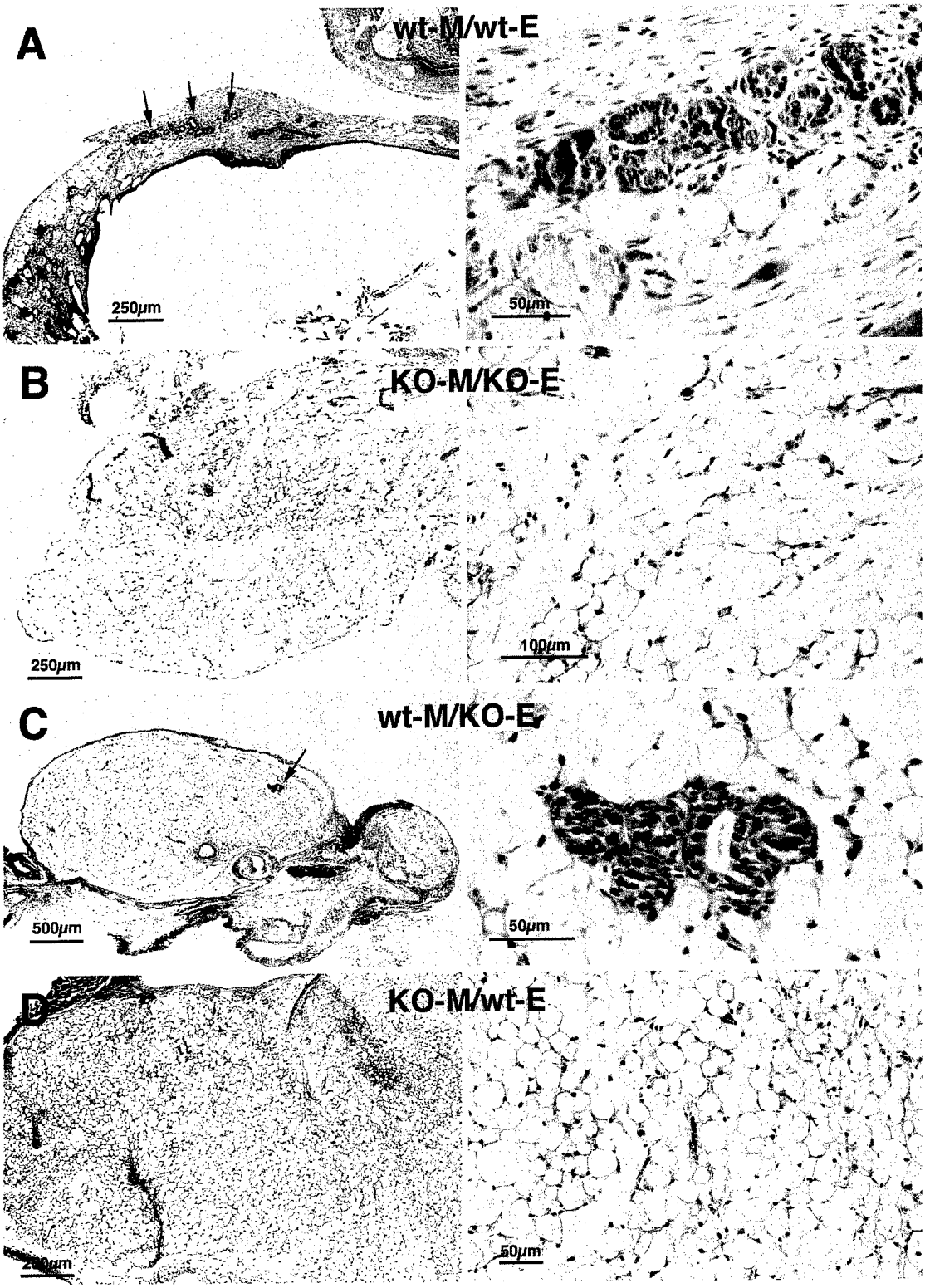

FIG. 7. Mesenchymal PTH/PTHrP receptor is necessary for the initiation of epithelial outgrowth. Mammary buds were dissected from wild-type and PTH/PTHrP receptor knockout embryos at E13, and the epithelium and mesenchyme were separated and then recombined in the four possible combinations and grown under the kidney capsule of recipient female mice for one month. Shown here are H&E-stained

to support epithelial cell outgrowth and ductal branching morphogenesis.

The first observation in support of this concept is that, during periods of active ductal morphogenesis, PTHrP and the PTH/PTHrP receptor are localized in epithelial and mesenchymal cells, respectively. Mammary ductal morphogenesis occurs in three distinct phases: during embryogenesis, during puberty and during early to mid-pregnancy. At each of these stages, we have shown that PTHrP is expressed in mammary epithelial cells and that the PTH/PTHrP receptor is expressed in mammary stromal cells. During embryogenesis, PTHrP mRNA was localized by *in situ* hybridization to the epithelial cells of the mammary bud and to the epithelial cells of rudimentary ducts as they progressed through the initial round of ductal branching morphogenesis from E16 to birth. The PTH/PTHrP receptor gene was expressed in the mesenchymal cells surrounding the mammary bud and in the mesenchyme that becomes associated with the branching epithelial ducts as they grow out into the developing mammary fat pad. This pattern of epithelial/mesenchymal expression was also observed during puberty. RNase protection analysis of PTHrP and PTH/PTHrP receptor expression demonstrated that PTHrP is found only within the portions of the mammary gland containing epithelial cells, but that PTH/PTHrP receptor mRNA is found both within the portions of the gland containing epithelium as well as within the fat pad alone. *In situ* hybridization demonstrated that this is due to the expression of the PTHrP gene in epithelial cells and its receptor gene in stromal cells. Furthermore, our *in situ* results demonstrated that, during puberty, both genes are most prominently expressed in terminal end buds. Interestingly, reminiscent of the fetal pattern of expression, we found that PTHrP mRNA appears to be localized to peripheral epithelial cells within the end buds which are known as cap cells (Daniel and Silberstein, 1987). PTH/PTHrP receptor message is found in the stromal cells immediately adjacent to the cap cells and enveloping the terminal end buds. Whereas we observed a low level of expression of the PTH/PTHrP receptor gene in the stroma of the fat pad and surrounding more mature ducts, we could not detect PTHrP mRNA within the epithelial cells of the mature ducts.

During pregnancy there was a low level of PTHrP mRNA expression in epithelial cells of the developing lobuloalveolar units, and it appeared that the PTH/PTHrP receptor gene was expressed throughout the stroma. These results are consistent with the findings of Rakopolous *et al.* (1992) who reported a diffuse, low level of PTHrP gene expression within mammary epithelial cells during pregnancy in rats. However, in our experiments, the levels of expression of both genes were just above the detection limit of our *in situ* hybridization technique and neither gene displayed an obvious pattern of expression as seen within the end buds of the adolescent gland. Despite this low level of hybridization, we are confident that PTHrP is expressed in epithelial cells and the PTH/PTHrP receptor is expressed in stromal cells during pregnancy for we obtained exactly these results using the more sensitive RNase protection assay. First, it is clear from Fig. 1A that both genes continue to be expressed on a whole gland level during pregnancy and, second, our analysis of epithelial and stromal cells isolated from pregnant mammary glands (Fig. 4) demonstrates PTHrP but no PTH/PTHrP receptor mRNA expression in epithelial cells and PTH/PTHrP receptor but not PTHrP mRNA in stromal cells.

The expression of PTHrP and its receptor in end buds in an epithelial/mesenchymal pattern is logical if PTHrP affects stromal function in a way that is important to the overall regulation of morphogenesis because the terminal end buds are the sites of active ductal growth and morphogenesis. During the embryonic development of the mammary gland, the initial round of branching morphogenesis occurs as a consequence of signaling between the mammary epithelial bud and its surrounding mesenchyme (Sakakura, 1991). Likewise, during puberty, epithelial–mesenchymal signaling at the terminal end bud influences the overall rate of ductal proliferation as well as the branching pattern of the growing duct system (Daniel and Silberstein, 1987; Silberstein and Daniel, 1987; Silberstein *et al.*, 1990). Therefore the recapitulation of the embryonic pattern of PTHrP and PTH/PTHrP receptor expression within the end buds suggests that PTHrP signaling to mesenchymal cells is most likely important to these processes during both phases of mammary gland development. This concept is supported

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sections through the resultant transplants after they were removed from beneath the kidney capsule. The left side of the figure represents low-power views and the right side represents the high-power magnifications of the same sections displayed on the left. Each transplant consists of a fragment of connective tissue containing varying amounts of fatty stroma, fibrous stroma, epidermal structures, and mammary epithelium. (A) Representative transplant resulting from wild-type epithelium recombined with wild-type mesenchyme (wt-MGE + wt-MGM). Note the mammary epithelial ducts (higher power view on right) located within a mixture of fibrous and fatty stroma. The structures at the lower left in the left-sided panel are hair follicles. (B) Representative transplant resulting from PTH/PTHrP receptor-knockout epithelium (ko-MGE) paired with PTH/PTHrP receptor-knockout mesenchyme (ko-MGM). Note that there are no epithelial ducts in this section, only fatty stroma. (C) Representative transplant resulting from wild-type mesenchyme (wt-MGM) paired with PTH/PTHrP receptor-knockout epithelium (ko-MGE). Note that epithelial ducts are present within the stroma (higher magnification on right), but that there are fewer ducts than in A. (D) Representative transplant resulting from knockout mesenchyme (ko-MGM) paired with wild-type epithelium (wt-MGE). As in B, note the complete absence of epithelial ducts. The arrows in A and C indicate mammary epithelial ducts. The scale bars in each panel demonstrate magnification as labeled.

by the results of our experiments in transgenic animals because disruption of the PTHrP gene results in the failure of the initial round of branching outgrowth during embryonic development and overexpression of PTHrP in the mammary gland in transgenic mice results in defects in ductular proliferation and branching during adolescence and pregnancy (Wysolmerski *et al.*, 1996; 1998).

We did not detect PTH/PTHrP receptor expression in epithelial cells by *in situ* hybridization during the time points we examined. This is in contrast to reports in the literature that have demonstrated PTH/PTHrP receptor expression in cultured myoepithelial cells and in several breast cancer cell lines (Birch *et al.*, 1995; Ferrari *et al.*, 1993; Seitz *et al.*, 1993). It may be that our *in situ* hybridization techniques cannot detect low levels of PTH/PTHrP receptor mRNA in myoepithelial cells, or perhaps the timing of PTH/PTHrP receptor expression in epithelial cells is not represented in our sampling. Alternatively, the presence of the receptor in myoepithelial cells may be specific to cultured or transformed cells. Our results cannot exclude that there may also be effects of PTHrP directly on some epithelial cells. However, these results clearly do demonstrate that the major location of PTH/PTHrP receptor expression is the stroma.

The second observation in support of stromal cells as a target of PTHrP's effects in the mammary gland is the ability of cultured stromal cells to express the PTH/PTHrP receptor, to bind amino-terminal PTHrP with high affinity, and to generate cAMP in response to PTHrP. These results, combined with our findings that freshly isolated mammary epithelial cells express the PTHrP gene but not the PTH/PTHrP receptor gene, represent a correlation of the *in situ* findings, *in vitro*, and underscore the concept that PTHrP, produced by epithelial cells, acts on stromal cells. This pattern is probably not unique to the mammary gland because dermal fibroblasts and lung fibroblasts have also been shown to respond to PTH and PTHrP, presumably via the PTH/PTHrP receptor (Rubin *et al.*, 1994; Shin *et al.*, 1997; Wu *et al.*, 1987). Furthermore, the ability of these cultured cells to retain their response to PTHrP now offers us an experimental system with which to begin to study the biological responses of stromal cells to PTHrP.

Our final observation in support of our working hypothesis is the demonstration that the expression of the PTH/PTHrP receptor in the mesenchyme is necessary for mammary epithelial cell survival and ductal morphogenesis. Using heterotypic tissue recombination and transplantation experiments, we have demonstrated that mammary mesenchymal cells require functional PTHrP signaling for these cells to support the survival and outgrowth of embryonic mammary epithelial cells. Unfortunately, experiments using tissues from PTHrP knockout embryos were uninformative, but mesenchymal cells taken from PTH/PTHrP receptor-knockout embryos were not able to support the survival or growth of either receptor-knockout or wild-type epithelial cells when these tissues were transplanted under the kidney capsule of recipient mice. These results mirror the failure of PTH/PTHrP receptor-knockout mammary buds to undergo the initial, embryonic round of ductal branching morphogenesis *in vivo* (Wysolmerski *et al.*, 1998). However, receptor-knockout epithelial cells were able to survive and initiate branching morphogenesis when recombined with normal mesenchyme, demonstrating directly that mesenchymal cells are critical targets of PTHrP's actions in promoting the outgrowth of the mammary epithelial bud. It should be noted that although receptor-knockout epithelium grew out and formed a rudimentary duct system when combined with normal mesenchyme, the growth of the resultant epithelial ducts was clearly not normal. These results may reflect the possibility that myoepithelial cells express a low level of PTH/PTHrP receptor which is below the detection limit of our *in situ* hybridizations (discussed above) but which is, nonetheless, important for the subsequent growth of the epithelial ducts following the initiation of branching morphogenesis. It may be that PTHrP signaling to the mesenchyme is sufficient to initiate branching growth during fetal life but that subsequent ductular proliferation requires a more complicated signaling cascade involving PTHrP's actions on both stromal and myoepithelial cells. To clarify this issue as well as the temporal requirements for PTHrP during ductular morphogenesis, it is likely that we will need to employ more sensitive localization techniques and to generate either conditional PTHrP-overexpressing or conditional PTHrP-knockout mice.

Despite the caveats noted in the previous paragraph, in the aggregate, it is clear that PTHrP's actions on mammary stromal cells have important consequences for ductal morphogenesis. Much work has demonstrated that the stromal mesenchyme plays a key role in determining the morphology of the epithelial duct system as well as in regulating the differentiation and functional activity of the mammary epithelium (Cunha *et al.*, 1995; Propper, 1973; Propper and Gomot, 1973). Recent experiments have suggested that the stromal regulation of epithelial function is complex. The stroma secretes growth factors such as KGF, HGF/SF, IGF1, and neuregulin which have been shown to regulate ductal morphogenesis (Hadsell *et al.*, 1996; Niranjan *et al.*, 1995; Ulich *et al.*, 1994; Yang *et al.*, 1995). In addition, the stroma contributes to the extracellular matrix, whose composition can have profound influences on epithelial behavior (Bissell and Hall, 1987; Sakakura, 1991). Finally, the stroma appears to be the principal source of matrix remodeling enzymes that also have been shown to have important effects on epithelial cell form, function, and survival (Alexander *et al.*, 1996; Sympson *et al.*, 1994; Witty *et al.*, 1995). All of these molecules are potential downstream stromal effectors of PTHrP's actions on epithelial development, and we are currently examining PTHrP's effects on their expression in our cultured mammary stromal cells. However, irrespective of the exact stromal response to PTHrP, our results underscore the truly reciprocal nature of the epithelial–mesenchymal interactions at play during the regulation of epithelial morphogenesis. Although stromal cells and their

products are critical to the regulation of epithelial form and function, it is clear that the epithelial cells participate in the regulation of their own fate, for without epithelial signals, such as PTHrP, the stromal cells are incompetent to direct epithelial morphogenesis.

In summary, our experiments demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal circuit that is necessary for mammary morphogenesis. Specifically, PTHrP produced by mammary epithelial cells must signal through the PTH/PTHrP receptor in mammary mesenchymal cells in order for the mesenchyme to support the initiation of mammary ductal morphogenesis. PTHrP has also been implicated in the development of other tissues that rely on epithelial/mesenchymal interactions for their development, including lung, teeth, and hair follicles (Philbrick *et al.*, 1998). Therefore, understanding PTHrP's role in regulating stromal cell function during the epithelial/mesenchymal interactions that govern mammary development should allow us to better understand the overall role of PTHrP in development.

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